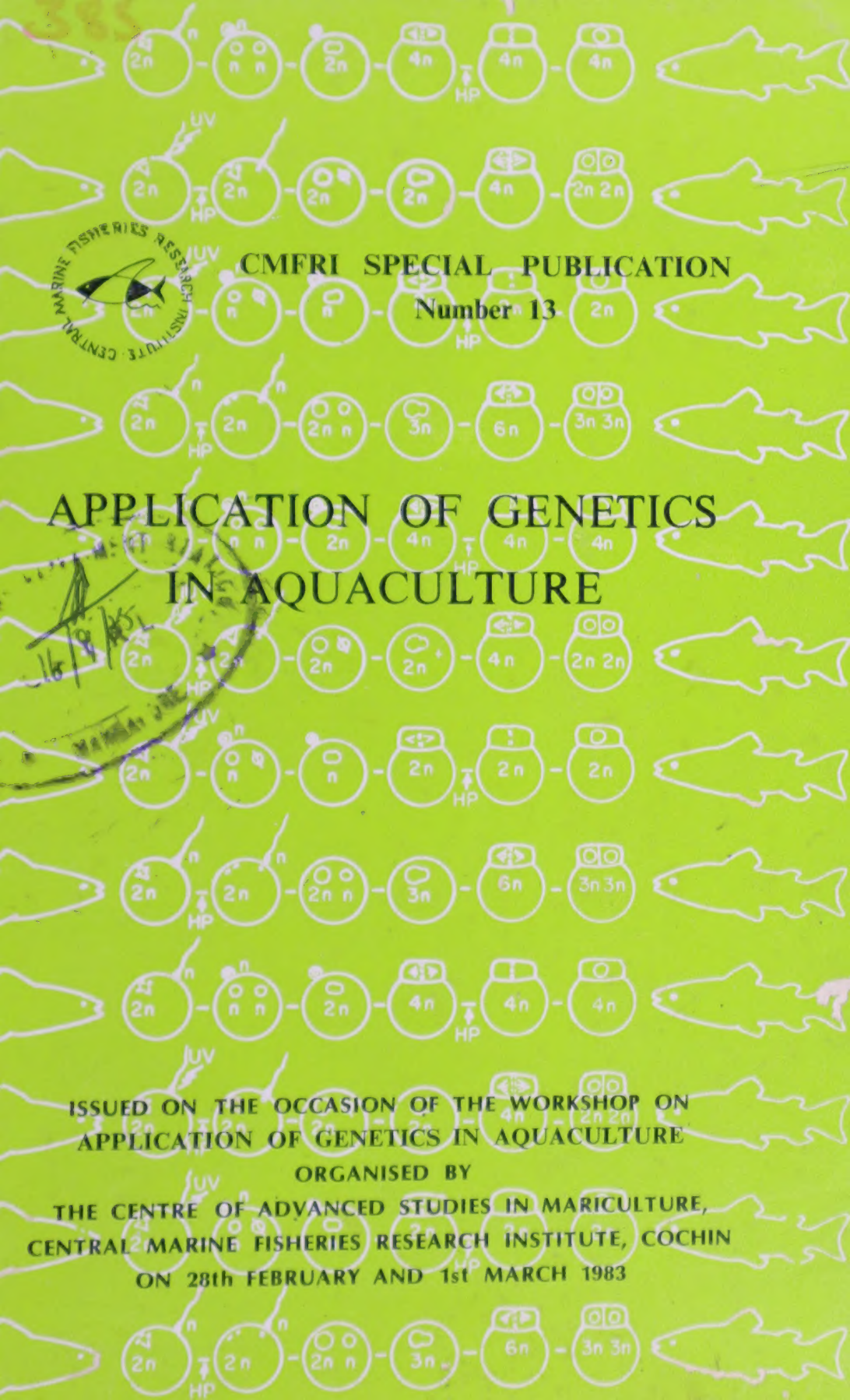

CMFRI SPECIAL PUBLICATION
Number 13

APPLICATION OF GENETICS IN AQUACULTURE

ISSUED ON THE OCCASION OF THE WORKSHOP ON
APPLICATION OF GENETICS IN AQUACULTURE
ORGANISED BY
THE CENTRE OF ADVANCED STUDIES IN MARICULTURE,
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE, COCHIN
ON 28th FEBRUARY AND 1st MARCH 1983

The CENTRE OF ADVANCED STUDIES IN MARICULTURE was started in 1979 at the Central Marine Fisheries Research Institute, Cochin. This is one of the Sub-projects of the ICAR/UNDP project on 'Post-graduate Agricultural Education and Research'. The main objective of the CAS in Mariculture is to catalyse research and education in mariculture which forms a definite means and prospective sector to augment fish production of the country. The main functions of the Centre are to:

— provide adequate facilities to carry out research of excellence in mariculture/coastal aquaculture;

— improve the quality of post-graduate education in mariculture;

— make available the modern facilities, equipments and the literature;

— enhance the competence of professional staff;

— develop linkages between the Centre and other Institutions in the country and overseas;

— undertake collaboration programmes; and

— organise seminars and workshops.

Under the programmes of the Centre, Post-graduate courses leading to M.Sc. (Mariculture) and Ph.D. are offered in collaboration with the University of Cochin since 1980.

APPLICATION OF GENETICS IN AQUACULTURE

PREPARED BY

V. J. BYE

Head, Fish Cultivation Group, Fisheries Laboratory, Lowestoft, U. K.

WITH ASSISTANCE FROM

A. G. PONNIAH

Central Marine Fisheries Research Institute, Cochin

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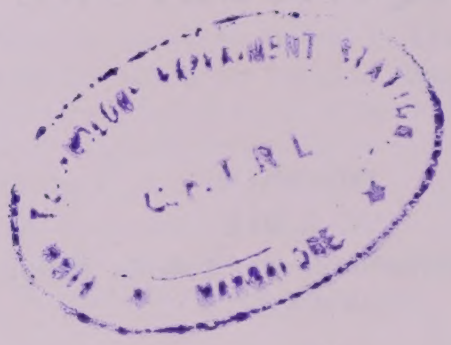
ON 28TH FEBRUARY AND 1ST MARCH 1983

(LIMITED DISTRIBUTION)



Published by : **E. G. Silas**
Director,
Central Marine Fisheries Research Institute,
Post Box No. 1912,
Cochin–682 018

PRINTED IN INDIA
AT PAICO PRINTING PRESS, ERNAKULAM, COCHIN–682 031

PREFACE

The exponentially increasing human population and the critical food supply have engaged the efforts of man in exploring the various avenues of food production and its augmentation to meet the requirement. This is being accomplished through the introduction of high yielding varieties of food and cereal crops, application of fertilisers and expansion of areas of cultivation in the agriculture front and introduction of quick-growing, quick-maturing and quality livestock along with the husbandary and managerial principles. In the fisheries sector, the production is being increased by means of modern technology of fishing, establishment of processing and other infrastructural facilities and recently through wider application of aquacultural activities.

The role of aquaculture in augmenting fish production and in area improvement is now well recognised. Considerable progress has also been achieved in the technology development for the culture of different organisms and in the corollary aspects of nutrition, controlled reproduction, seed production and mortality control. Nevertheless, one of the major problems encountered by the aquaculturists is the selection of productive or economic species or strains that could be successfully cultivated and subsequently domesticated in different environmental conditions. It is in this context, the genetic approach in aquaculture to the problems has assumed great importance. Besides, there is also an increasing awareness to understand the genetic make-up and variability of the wild as well as farmed fish stocks for the management of genetic resources for genetic improvement.

Although the basic science of fish genetics is similar to animal genetics and appreciable progress has been made in recent years in selection, inbreeding, hybridization and sex control of certain

cultivable species, the application of the technologies or the knowledge gained in practical aquaculture programmes has been limited or little accomplished. It is also realised that there is distinct difference between fish and animal genetics and that aquaculture candidate species present greater genetic variability. These facts point out to the urgent need to undertake both basic and applied research on different aspects such as the biological characteristics of the genetic resource of the unit stocks of fishes, population genetics, selection, breeding schemes, inbreeding, hybridization, utilisation of heterosis, chromosome engineering, sex control and cryopreservation of gametes.

In India, information of the genetic make-up or genetic variability of marine finfishes and shellfishes is meagre, although success has been achieved in controlled breeding and hybridization of certain freshwater fishes and in the development of breeding technology of shellfishes, particularly of the penaeid prawns, and molluscs such as mussels, edible oysters and pearl oyster. Realising the importance of application of genetic principles in the emerging aquaculture programme in our country and the long-term needs for preserving the genetic resources of the cultivable organisms, it is identified as a priority field for research and development.

While considering the research strategies to be taken up in the field in our country, it is stressed that the advantages now available with the naturally evolved genetic make-up of the wild stock to maintain the breed purity and to prevent the inbreeding depression should be taken note of. Further, it is pointed out that the genetic research in our country where tropical environment prevails, could gain tangible results due to the biological features of the species in having amenable breeding strategy, faster rate of growth and shorter generation time as compared to the species that live in temperate environment exhibiting relatively protracted breeding period, slow growth rate and larger life span.

One of the major constraints in the promotion of genetic research on marine fishes and shellfishes in our country is the lack of trained fish geneticists and standard manuals containing research

methodologies and techniques. In order to fill up this gap, the **Centre of Advanced Studies in Mariculture** under its programme on consultancy, invited Dr. V. J. Bye, Head of Fish Cultivation Group, Fisheries Laboratory, Lowestoft, U. K. to provide technical advice in the subject. During his consultancy period with the Centre, a Workshop on 'APPLICATION OF GENETICS IN AQUACULTURE' was organised under his leadership and on the occasion of this workshop, the present manual prepared by him in association with Dr. A. Geethanand Ponniah, Scientist of the Institute was issued.

This manual was prepared with a purpose to provide a base on which the research scholars or the fresh research workers could obtain basic information necessary for fish genetic studies and to understand the complex processes involved in the subject. In part 2 of the manual are given the basic techniques which it is hoped, would help the research worker to plan his programme of study on an established base and to modify them to suit his test fishes. I wish to express my gratitude to Dr. V. J. Bye for his contribution and diligent task of preparing the manual as well as for demonstrating the various technologies employed in fish genetic studies to the participants of the Workshop. I also wish to thank Dr. A. G. Ponniah who was the counterpart to Dr. Bye and intimately associated in the preparation of the manual as well as in the conduct of the Workshop. I appreciate the tedious task of editing the manuscript for the press accomplished by Shri K. Rengarajan, Scientist of this Institute.

E. G. Silas
Director
Central Marine Fisheries
Research Institute

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PART 1

1

THE ROLE OF GENETICS IN AQUACULTURE

Modern high-yielding plants and animals used in agriculture are derived from 'wild' stocks but their productivity has been enormously increased by a combination of genetic and husbandry methods. Although husbandry has been considerably improved in aquaculture there has been very little genetic improvement. This is partly because of the much shorter history of aquaculture, which for most species is less than a century, and partly because rearing aquatic organisms, which frequently have complicated life cycles, is intrinsically more difficult than rearing land plants and animals. All farmed aquatic animals, with the possible exception of carp, are genetically indistinguishable from the wild populations from which they were captured but there is considerable scope for genetic improvement and for the application of specific genetic manipulations which will significantly improve the productivity of aquaculture (Fig. 1).

This handbook will consider the range of genetic techniques which are available to the aquaculturist and provide practical details of some of them. It is important that genetic programmes should start simultaneously with the domestication of new species in order to benefit from the maximum population heterogeneity

Genetic selection for external features can be very effective and carp and other decorative fish have been selected for colour

or fin and body shape over hundreds of years, often with dramatic results. However, such superficial features are of limited interest to the food producer who would prefer to use selection to improve performance characteristics such as growth rate, food conversion efficiency, disease resistance, fecundity, egg-size and so on. The fundamental concept of selection is that like begets like but there is always some variation within a group of individuals from which preferred progenitors can be selected to derive the domesticated line of animals or plants towards that combination of characteristics which are considered desirable. So selective improvement is achieved by choosing as parents those organisms which most closely resemble the ideal. Unfortunately although selective improvement has been held to be the solution to all defects in cultivated organisms its realization is rarely as simple as its conception.

Any observable or measurable characteristic is a product of the genetic constitution of the individual and its environment but the relative contributions of genotype and environment vary considerably for different characteristics and under different conditions. With carefully controlled experiments it is possible to estimate the genetic and environmental components of the variation of a character and calculate its heritability. Only those features with high heritability, whose variation is atleast 10% under genetic control, can be easily modified by selection.

Selective breeding in aquaculture has yet to produce substantial improvement. The best trials are those conducted by the Israelis over five generations with common carp. They were unable to demonstrate any significant improvement in growth rate and suggested that this character was already close to its limit. However, recent experiments, in particular with salmonids and molluscs suggest that some selective improvement of growth may be possible.

Research in the U.S.A. on salmonids in which substantial improvements in growth rate, fecundity, size at maturity and disease resistance have been reported are impossible to evaluate, because unselected control lines were not maintained. The

spectacular gains are more easily attributed to improved husbandry than they are to selection. This emphasises the importance of employing adequate controls in all genetic experiments.

The selection of disease resistant strains appears to be a more immediately productive line of research. Both Israel and Russia have developed disease resistant strains of carp and different strains of salmonids have been shown to vary considerably in their susceptibility to a range of disease organisms. Even in the American oyster it has been possible to breed strains which are upto nine times more resistant to specific diseases than are the wild stocks. However, the possibility of the pathogen evolving as fast as the selection process must be guarded against.

Many other characters of economic importance such as fecundity, egg size and time of spawning are likely to be amenable to selection but a more immediately productive approach may be a survey of the wide range of existing domesticated strains and natural populations for desirable traits. Considerable variations are apparent in fish and shellfish and it is probable that initially the evaluation and utilization of these differences will be easier than the analysis of selection responses.

Hybridization between different species rather than between different strains of the same species has been of limited value in agriculture but may have more potential in aquaculture. Fish hybridize relatively easily and although most hybrids are inviable, or far less fit than the parents, the occassional one does show hybrid vigour or a particularly useful combination of characteristics – often including sterility. One of the most successful hybrids has been between members of the sturgeon family where the artificial cross between the one pound freshwater sterlet and the one ton marine beluga resulted in a hybrid which exhibited excellent growth in freshwater.

Inbreeding and crossbreeding of inbred lines is another potentially valuable genetic technique. Although the inbreeding which arises from the mating of closely related individuals is normally accompanied by a loss of vigour in the population, when two

genetically different inbred lines are crossed they often produce very vigorous offspring. Inbred lines are genetically uniform and of no intrinsic value. Their value derives from the crossing of

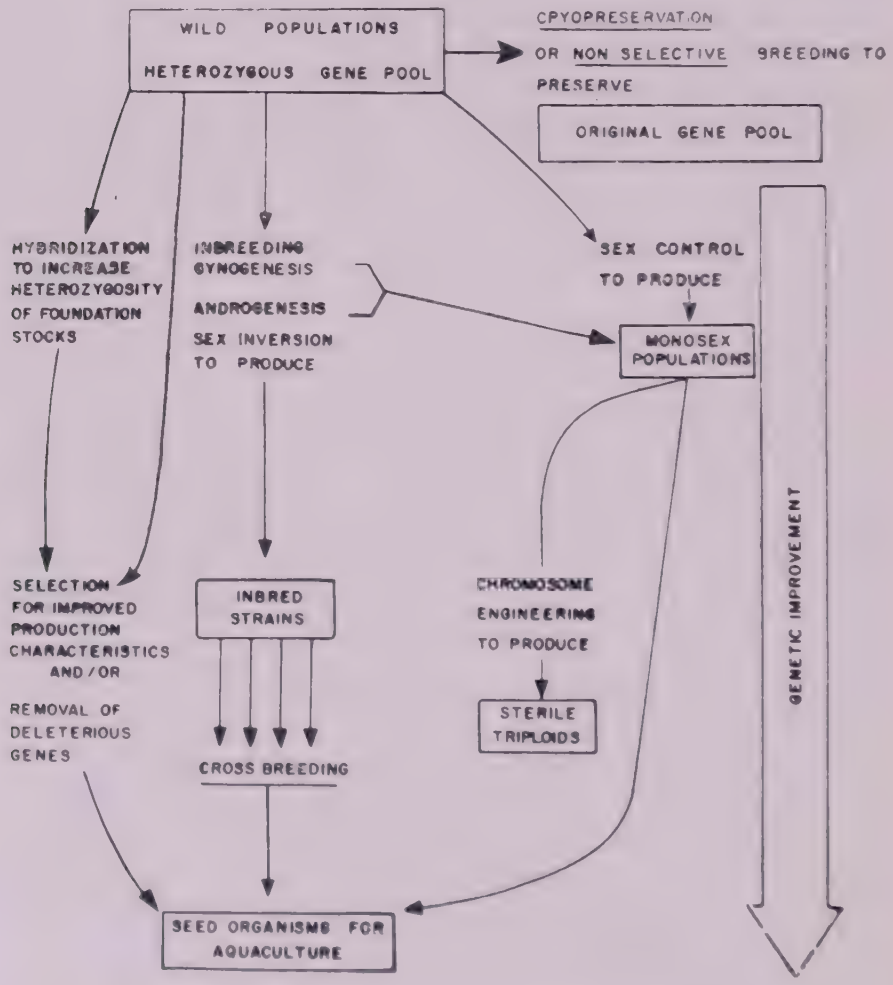

Fig. 1. Genetic improvement of aquacultural species.

two different lines with the production of F1 hybrids which exhibit good growth, high survival and uniform production characteristics. These techniques have been employed extensively in agriculture and are responsible for much of the enormous increase in productivity which has occurred over the last 40 years.

The high fecundity of most fish and shellfish has already resulted in a considerable degree of unconscious inbreeding and the economic losses caused in inbreeding depression are probably far more extensive than is commonly realised. The Israelis have demonstrated that production of F1 hybrid carp has considerable potential and preliminary work with salmonids indicates that F1 hybrids are ideal production animals. Full evaluation of the technique awaits rigorous testing under commercial conditions.

Sex control : Sterility is often associated with interspecific hybrids but the valuable characteristics of sterility and monosexuality can also be produced by other methods. The aquaculturist normally only wants to see sexual maturation in his broodstock because in other organisms it diverts from normal feeding, growth and conversion efficiency, increases aggressive behaviour and disease susceptibility and damages the appearance and flesh quality. Most salmonids cannot survive the stresses of sexaul maturation in salt water. In many organisms these problems are most pronounced in males and tend to occur earlier in them. Trials in England with rapidly grown rainbow trout demonstrated that as many as 40% of the males mature at one year and growth depression starts well before and persists after the spawning season. Sexual maturation can decrease productivity by upto 20% in small rainbow trout and the losses mount when larger fish are produced.

The obvious ways to eliminate the adverse effects of maturity are to produce stocks which consist entirely of females or of sterile animals and considerable success has been achieved with both approaches. Sex can be modified by the administration of sex steroids to larval and juvenile organisms and sterility can be induced by hormone treatment and surgical or autoimmune castration.

Chromosomal engineering : The artificial modification of the chromosome set of an organism, also permits the production of monosex and sterile individuals. In addition gynogenesis provides a method for the rapid production of inbred populations for use in crossbreeding programmes.

Gamete manipulation and storage : Genetic improvement of aquatic organisms is facilitated by the relative ease with which *in vitro* fertilization can be achieved. External fertilization tends to require gametes capable of survival outside the body and these are usually also capable of surviving the procedures applied to them by the geneticist. A variety of techniques have been developed for the manipulation of gametes and for their short and long term storage outside the parent's body.

Cryopreservation of spermatozoa has been relatively successful and provides valuable long term storage which permits crosses between strains or species whose spawning is geographically or temporally separated. It can also provide a bank of valuable genetic variation or a source of unselected material with which to compare the performance of improved strains.

2

SELECTION

The aim of a breeder is to get maximum profit possible from the organism he is cultivating. This he can achieve by improving the genetic worth of the stock by selection. Selection can be based on a single desirable trait or combination of such traits.

Selection should begin simultaneously with the domestication of a new species while the widest gene pool is still available. Once domestication starts there is inevitable gene loss. There are prerequisites before a selection programme can be established. These include:

1. The breeding goal *i.e.* the trait under selection has to be defined as specifically as pos ible.

2. The entire life cycle of the animal should be under control.

3. It should be possible to hold and individually evaluate a number of generations in more or less identical rearing systems.

4. The individuals within a tested population should be identified, by means of external tags or distinct biochemical genetic markers.

5. For the traits that are to be selected, their relative economic value should be established.

6. For the selected traits, the phenotypic (VP) and genetic variances (VG), heritabilities (h^2) and genetic and phenotypic correlations should be known.

$$VP = VG + VE$$

where VE is the paratypic or environmental variance.

The immediate breeding goal may vary with different species and under different conditions depending on production aims. Nevertheless some traits such as growth and survival are of obvious general importance and they will usually be selected for the initial breeding goal. The most common goals are to evolve breeds which:

1. utilize food more efficiently for growth *i.e.* have higher food conversion efficiency;
2. can eat a maximum of the natural food available;
3. have high survival rates particularly in larval stages;
4. have resistance to parasitic and infectious diseases;
5. are tolerant to unfavourable environmental conditions such as low oxygen levels, extreme temperatures, fluctuating salinity, low pH, etc.;
6. have superior meat quality and smaller proportions of inedible body parts so as to improve marketability.

It is often difficult to obtain selection improvements in traits associated with reproduction. They have frequently reached a selection plateau and have low heritability.

Most characteristics of economic importance for aquaculture are measurable (metrical) and show individual variation within a population. This usually takes the form of a normal distribution

(Fig. 2) which mathematically is the probability combination of a large number of random events. The magnitude of a metrical character depends on a multitude of factors, many genes and numerous environmental influences, so it approximates to a random event. The normal curve is characterized by:

$$\frac{1}{\sqrt{2\pi}\,\sigma}\, e^{-\frac{1}{2}\frac{(v-\bar{X})^2}{\sigma^2}}$$

where $\bar{X}$ = mean; v = individual value;

n = number of individuals;

σ = standard deviation.

The square of the standard deviation σ^2 = the variance

Dimensional values are abolished if the standard deviation is expressed as a percentage of the mean value. This is the coefficient of variation (cv).

$$cv = \frac{100 \cdot \sigma}{\bar{X}}$$

The response (R) to selection over a unit time is dependent on many parameters (Fig. 2). They are the heritability, the selection intensity, the phenotypic standard deviation, the generation time and the genetic correlation if more than one trait is involved. Response can be calculated from the equation.

$$R = \frac{S \cdot h^2}{L} = \frac{i \cdot \sigma \cdot h^2}{L}$$

where R = response. Change in population mean from generation to generation.

S = selection differential. Difference between mean of selected parents and population mean.

i = selection intensity. The percentage of the population chosen as parents of the next generation. Because of the high fertility of aquatic animals a very strong selection pressure upto 0.1% (1:1000) can be applied.

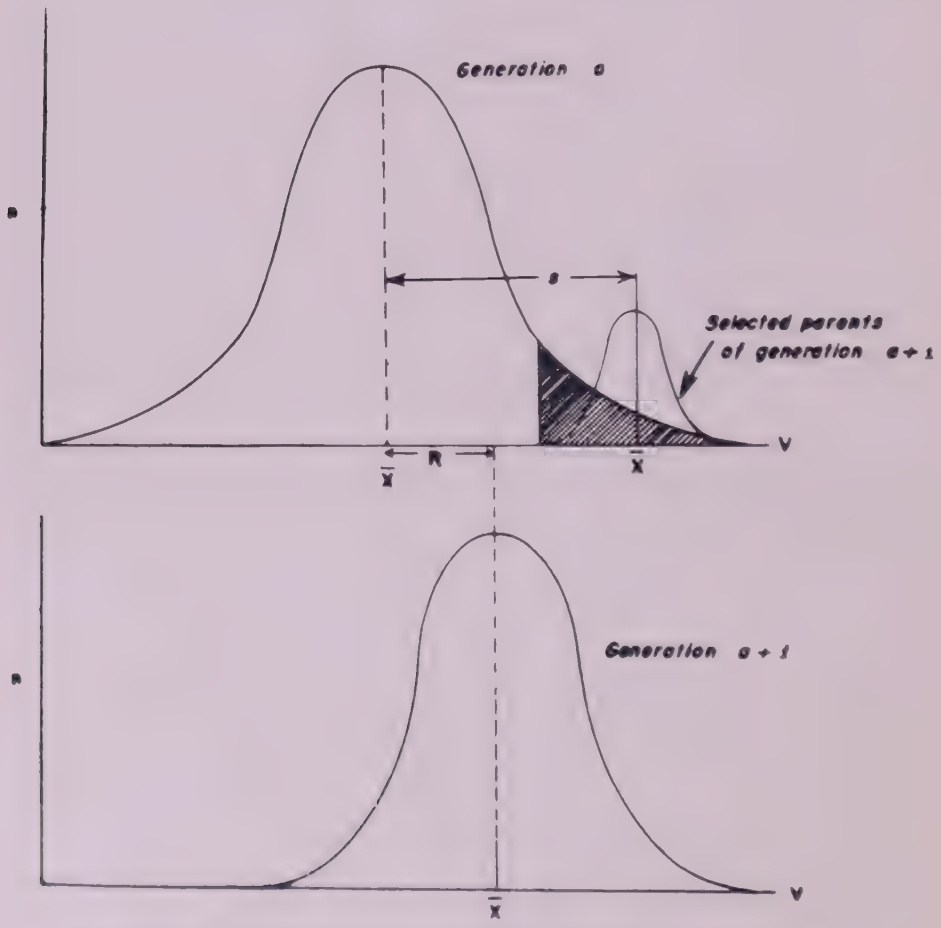

Fig. 2. Distribution of a character within a population.

h^2 = heritability. This is the proportion of additive genetic variation in the total phenotypic variation. If heritability is high and close to 1.0 most of the variation in a trait is heritable and selection will be very effective. If environmental factors have caused most of the variation this value will be low and

if h^2 is zero no genetic gain can be obtained by selection. There are many ways of estimating heritability; two of the most commonly used are sib analysis or from the observed selection response.

$$h^2 = R/S$$

To adopt a suitable method of selection, the estimate of coefficient of heritability is essential.

σ = phenotypic standard deviation. This should be sufficiently large and could be variation in repeated measurements at different times in life of an individual.

L = generation interval. The time in years for individuals to reach sexual maturity and breed. Decrease in the generation interval will directly increase the response to selection over a unit time.

There are a variety of selection methods and the most commonly used are:

1. Mass selection

This is also known as character or individual selection. The procedure involves the measurement of characteristic or performance of the potential parents and then the selection as parents of those with records closest to the breeding goal. This type of selection is effective of characters whose heritability is in the range approximately 0.25–1.0 and which can be measured easily in the potential parents. Historically, mass selection has been the main method used in fish breeding but in general success has been limited for production characteristics. This is primarily because the fundamental requirements for selection have not been known or applied. Particular problems have included:

i. Poor correlation between growth rate and age. Selection for high fingerling size in fishes may not improve the

overall growth rate to marketable size or selection at marketable size may not improve the growth rate at fingerling size. This is because growth at different ages is influenced by different factors and heritability for size often increases by 2 to 3 times after the fingerling stage.

ii. Inadvertent selection of aggressive individual rather than those with optimum conversion ratios. The economically important differences in food assimilation and conversion ratios become of secondary significance when the aggressive individuals with better food gathering qualities are selected. Obviously if a population is composed entirely of aggressive individuals its overall performance will decline.

iii. The reduction of genetic variability due to inbreeding which limits the scope for selection.

Mass selection can be more effective if unrelated populations are used to start the breed thus producing a heterogeneous gene pool from which to select.

2. Family selection

When the heritability of a character is low in the range 0 to 0.25, the phenotype of an individual is a relatively inaccurate measure on which to base selection. It is more effective to base selection on family performance. This procedure requires multiple crossings between selected parents, comparative evaluation of the progeny and selection of progeny from the best families for further raising. From the start, the rearing conditions of all families being compared must be as similar as possible to minimize environmentally induced interfamilial variability.

3. Sib selection

This is a variation of family selection and is useful for selecting for biochemical, physiological and nutritional qualities. It requires

a large number of individuals in each generation from which selection can be made based on the performance of full or half sibs of the individuals selected for breeding.

4. Selection by progeny testing

A prerequisite for selection by progeny testing is artificial fertilization of eggs. The progeny are first compared under laboratory conditions and then in ponds. The raising of progeny must be repeated for several spawnings. In selection programmes careful attention has to be paid to (a) correlated changes in other characters (b) genotype - environment interaction. It is usually seen that the selection for one character leads to correlated changes in other characters. This is because many characters are genetically correlated as a consequence of pleitrophy and linkage. If the correlation is due to pleitrophy there is no possibility of changing the relationship between two characters. However, if linkage is the cause, then the relationship will alter when appropriate crossovers take place. The genetic correlation between characters is within the limits + 1 to - 1 and can be estimated when two or more characters are measured in individuals used for the estimation of heritability. This must be followed in a selection programme because correlated changes occur in other productive characters.

5. Selection for more than one character

It is rarely possible to select for only one character since most populations will require improvement in a number of traits. There are three main methods of multiple character selection.

a. Tandem selection

This procedure is the simplest one of selecting for one character for several generations and then changing the objective of selection and selecting for the second character for a further several generations. This method is rarely used because it takes so long; because simultaneous change of characters is usually required and because characters may be inversely correlated.

b. Selection by means of independent culling levels

This procedure involves setting independent thresholds of brood stock selection for different characteristics and then taking as parents only those individuals which fall within both thresholds (Fig. 3). This method is easily operated and widely used in agriculture.

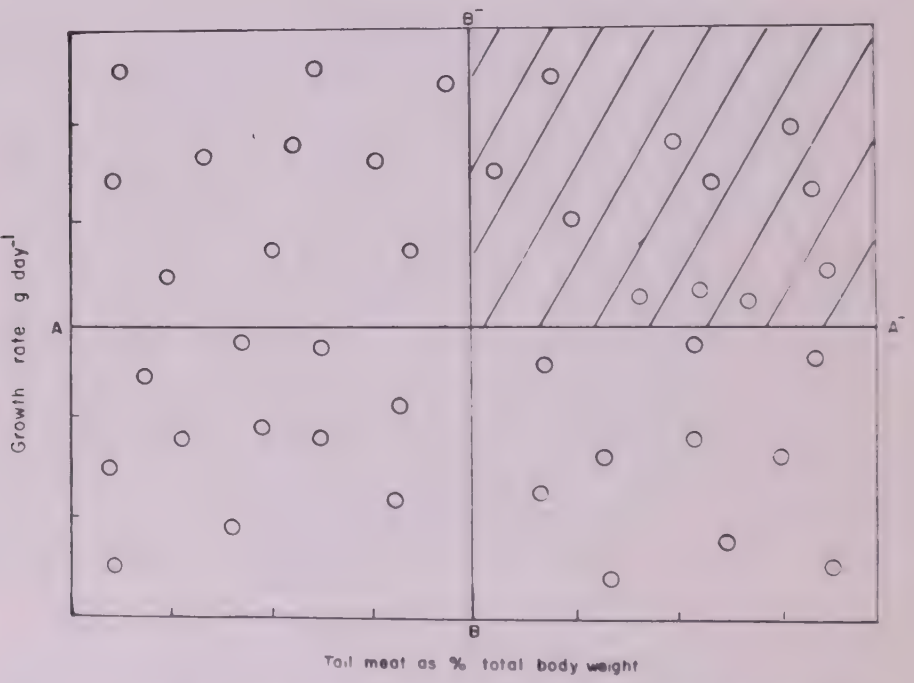

Fig. 3 Multiple selection by independent culling levels.

Note: culling level for growth rate is shown by line A $\bar{A}$ and for dressout value by line B–$\bar{B}$. Only those individuals falling withinthe hatched area will be selected as brood stock.

c) Selection by means of an index

This method involves the combination of measurements of two or more characters into a single value for each individual. The index is then used as the criterion of selection. However, calculation of a single value is not easy, particularly when more than two characters are involved and sophisticated computation facilities are required.

Both theoretically and in practice it has been found that tandem selection is by far the least effective of the three methods, whilst index selection is about 10% more effecient than independent culling.

The response to different selection methods depends on the heritability of the trait under investigation (Fig. 4) and the best response can frequently be obtained by a combination of Family and Mass selection.

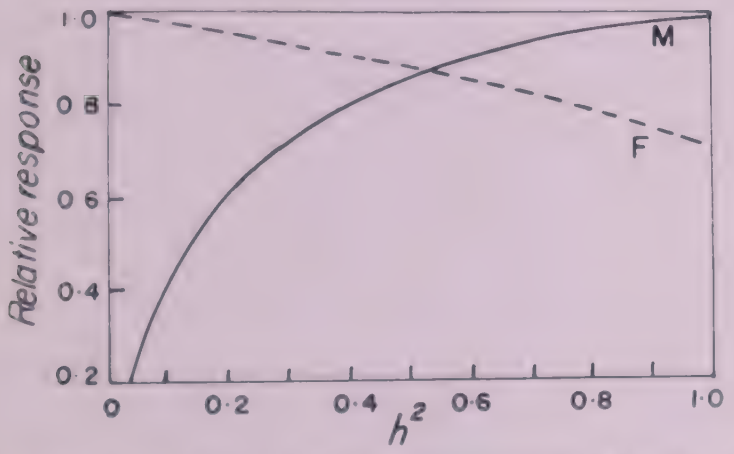

Fig. 4. Relative merit of Family (F) selection compared with Mass (individual) (M) selection (Falconer, 1960).

In a planned selection programme the environmental circumstances under which the programme will be conducted and results of the programme applied, has to be decided. It must also be established whether the heritability of the selected character differs between the different environments. The greatest rate of genetic change will be achieved in environments which induce the higher heritability. However, the applicability of the programme frequently depends on selected animals performing equally well in other environments *i.e.* there should be no strong genotype-environment interaction.

3

HYBRIDIZATION

The term hybridization is usually used to denote the process of crossing two different species. However, intergeneric or intra-specific hybridization can also be achieved. In the latter case genetically or geographically distinct populations of the same species are crossed. Hybridization is usually performed to obtain the associated advantages of heterosis, commonly known as hybrid vigour. Heterosis can be defined as the additional performance if any, shown by the first generation (F1) of crossbred progeny above the mean performance of either set of parents. If heterosis is observed in the F1 generation then hybridization is profitable, but in general most hybrids are less viable or less productive than their parents. The success of hybridization is unpredictable and can only be checked by evaluating the progeny. Two factors help to predict a successful hybridization.

i. Knowledge of karyotype and

ii. Standard procedures of artificial fertilization.

A knowledge of the karyotype of two species to be hybridized is not absolutely essential, but a similar number of chromosomes indicates a better chance of hybridization. As a general guideline the smaller the number of chromosomes, the more important it is for the hybridizing species to have equal numbers of chromosomes.

In species with a large number of chromosomes, viable hybrids have results between species with dissimilar karyotypes.

In all cases hybrids have a karyotype representing the sum of haploid numbers of the two species.

Most hybridizations are carried out by artificial fertilization since behavioural or geographical barriers prevent normal matings. So reliable techniques of artificial fertilization are important for hybridization. A satisfactory system of sperm cryopreservation also facilitates a hybridization programme. If mating or artificial fertilization is successful then hybrid survival depends on gametic compatability and viability of the hybrid genome.

The same hybrid cross can produce very heterogenous results and careful evaluation is required. There are two main methods of reducing this variability.

i. When two species are crossed a control cross within each species should be carried out. The control animals must be reared in the same environment as the hybrids to permit a reliable estimate of hybrid performance. In this way it is possible to reduce the variability due to the quality of parental gametes and to environmental effects.

ii. Care should be taken to always use the same strains in interspecific hybridization to avoid the influence of intraspecific variability.

Hybrids can be fertile or sterile. Before introduction of a hybrid in to the natural environment, this must be verified by keeping hybrids and their control crosses until sexual maturity. Fertility can then be assessed by the presence and degree of gametogenesis compared to the controls. The gametes produced must be tested in a fertilization trial.

A successful hybridization confers some advantages and call attention to certain precautions. Besides hybrid vigour, hybridization provides some information on the genetic similarity between

the species. If the hybrid is totally sterile and if sexual maturation in the parent results in delayed growth or poor flesh quality the hybrids provide additional advantages. There is also no danger of sterile hybrid replacing the parent species in the natural environment. If hybrids are fertile this aspect needs careful control. Indiscriminate hybridization can result in contamination of the gene pool of a species with damaging effects as has happened in *Tilapia* species.

Hybridization usually produces an extensive gene pool with pronounced heterozygosity and this forms a valuable basis for selection programmes. Hybrids usually have to be sold under a new name and if they differ morphologically from the preferred parent species, there can be considerable consumer resistance.

4

INBREEDING AND CROSS BREEDING

Inbreeding occurs when mates are more closely related by ancestory than the average relationships of all individuals in the population. Matings involving full or half sibs, offspring and parent, or even cousins can be termed inbred matings. The highest values of inbreeding can be obtained by mitotic gynogenesis (see chapter on Sex Control and Chromosomal Engineering). For the aquaculturist inbreeding has both negative and positive aspects.

Inbreeding leads to decreased viability and retardation of growth, the so called inbreeding depression. This depression is mainly due to an increase in the number of homozygous genes, particularly when harmful recessive genes become homozygous. In cultured aquatic organisms which often have very high fecundities brood stocks are frequently small and there is a greater chance of inbreeding.

Inbreeding is useful in that, selected traits are stabilised due to increased homozygosity and their expression may be augmented. It is also possible to derive a high degree of heterozygosity when two unrelated inbred strains are crossed. The animals derived from such cross breeding can be used for commercial production since they are fast growing, have high survival and exhibit uniform

production characteristics. When intensive selection has been carried out for a long time, it is impossible to maintain high heterogenity of the population. In such a situation cross breeding with a distant strain or from a genetic reserve pool of animals maintained without inbreeding will restore the level of heterozygosity and if the F1 hybrid is crossed back into the selected line much of the selected gain will be retained.

The degree of inbreeding can be calculated from the size of the population used as broodstock at each generation.

The coefficient of inbreeding F is the probability that two genes at any locus are alike by descent.

It is assumed that there is no inbreeding in the founder generation so that:

$Fo = 0$

N = population size of brood stock

Then at the first generation $F1 = \frac{1}{2}N$. Inbreeding accumulates from generation to generation so that at generation n

$$Fn = \frac{1}{2N} + (1 - \frac{1}{2N})\ Fn - 1 \text{ or}$$

$$Fn = \left[1 - (1 - \frac{1}{2N})^{n} \right]$$

However, breeding populations rarely conform to the ideal. Not all animals contribute equally to the next generation and the number of males and females may not be equal. It has been found useful to employ the concept of effective population size, Ne, which is defined as the number of individuals, which if bred in the manner of the idealized population, would give rise to the same rate of inbreeding as the actual population under consideration.

$$Ne = \frac{4\ (\ N\ \sigma^{\prime}.\ N\ \varphi\ )}{N\ \sigma^{\prime} + N\ \varphi}$$

With limited numbers of individuals Ne is highest when the sex ratio is approximately 1. When the sex ratio is highly skewed Ne can be approximated by the least numerous sex.

For example if 2 males are used to fertilize 110 females:

$$Ne = \frac{4\ (2 \times 110)}{112} = 7.85$$

effective population size is less than 8 although actual population size is 112.

It has been calculated that in order to prevent significant inbreeding no stock should be founded or perpetuated using less than 30 males and 30 females, chosen at random, for each generation. Ne should be 40 to 60.

The actual number of parents is also important in order to maximise the variety of alleles within the population. The greater the heterozygosity of a population the better the chance of selective improvement. The presence of an allele in a population is probably more important than its frequency since the latter can be adjusted by selection.

5

COLLECTION OF BASIC GENETIC DATA

The raw material for a geneticist to work upon and improve a species is the variation naturally present in the productive traits of individuals in a population. Little genetic gain can be achieved in traits where there is limited variation or in which the variation is mainly caused by the environment (see chapter on Selection). Therefore, even when a genetic programme is not in progress, collection of basic genetic data during breeding work will be helpful, since unconscious inbreeding and or selection may occur and this effect can be pronounced in animals with high fecundity. When improvements in husbandry are exhausted and attention is focussed on the possibility of genetic gain such data will be extremely useful in designing a programme. The collection of basic genetic data for genetic improvement will be more meaningful if it takes into account the following points:

i. Data on all parameters relating to production such as fecundity, hatchability, survival, growth rate and where possible conversion efficiency, age and time of spawning etc. should be kept for each brood and preferably for the parents as well.

ii. The data should be recorded at different ages

iii. There should be no mixing of broods.

iv. Where possible the individual variation within a brood must be ascertained by tagging.

v. Since environment by itself and in interaction with genotype produces most of the variation, data on relevant environmental parameters should be recorded.

vi. Ideally a sample of each brood should be evaluated in standard rearing conditions which can also be duplicated for other generations.

6

BIOCHEMICAL GENETICS

Introduction

The genetic analysis of aquatic organisms either for fisheries population studies or for aquaculture is most easily accomplished by controlled crosses between selected parents followed by progeny testing. This is not, however, always possible, therefore the techniques of biochemical genetics offer an alternative method of analysis.

Historically, both population and aquacultural genetics were based on superficial phenotypes or metrical characteristics having complex polygenic inheritance and considerable environmentally induced variation. But, in addition to these obvious characteristics there are genetically determined differences at the protein level which are much less subject to environmental influence. The genetic code of DNA is translated into proteins and there are frequently subtle, non-functional variations in the structure of homologous proteins in different individuals originating from small variations in the genotype. These phenotypes, which can be detected by biochemical means, in particular electrophoresis, are relatively easily studied in both natural and cultured populations. This provides a rapid and efficient way of obtaining much of the basic genetic information crucial to the genetic improvement of aquaculture.

Protein structure

The structure of a protein has 4 main characteristics:

1. Amino acid sequence;

2. The structure of the amino-acid chain (usually an $\propto$ helix):

3. The folding and bonding of the $\propto$ helix, and

4. The number of polypeptides in the protein which are either same or different. The protein can be either **Monomeric** or **Multimeric** with the latter being either **Homo** multimeris or **Hetero** multimeris. For example, Glutamine synthetase has 12 identical peptide subunits making it homomultimeric, whereas Lactate dehydrogenase is a tetrameric heteromultimer containing two different polypeptides produced at different loci. This will produce 5 different types of LDH molecule with the same functional activity but which are biochemically identifiable. Such alternative enzyme forms derived from different loci are **Isozymes**, whereas multiple enzyme molecules encoded by alternative alleles at a single locus are **Allozymes.** These terms are occasionally used interchangably, or allozymes are termed segregating isozymes.

Most proteins, in particular those with enzyme function, contain amino acids with electrically charged side chains. Arginine, histidine and lysine are positively charged while aspartate and glutamate carry a negative charge. Thus virtually all proteins have a net charge depending on the relative proportions of amino acids, unless they are at their isoelectric point, the pH at which the net charge is zero.

The basis of electrophoretic separation is that proteins of different net charge and different molecular size will migrate at different rates within an electric field.

Electrophoresis

A buffer of appropriate pH is used to soak a supporting medium, usually a gel, on which is placed a protein solution. When a current is passed through the buffer the different proteins migrate towards the opposite charge pole at a rate which is proportional to the magnitude of their charge and are thus gradually separated. The electric field is removed after several hours and the separated protein bands are then stained under appropriate conditions. The pattern of stained bands is termed a **Zymogram**. Proteins with marginal structural differences are easily distinguished. Obviously, proteins which have the same net charge and molecular size will migrate at the same rate and will not be separated. So the common assumption that a single band contains the products of a single non-varying locus is not necessarily valid.

Sources and extraction of proteins

For electrophoretic separation, proteins must be in solution. These can be natural solutions such as haemolymph, blood plasma, milk, semen, humoral fluid, etc. or aqueous extracts of tissue proteins. Extracts are often made from muscle, liver and eyes and in the former case can be obtained without killing the animal. Crustacea can be sampled non-lethally by removing a walking leg. The pH and ionic concentration of the buffer used, permits a differential extraction of proteins. With larvae, spat or other small organisms it is necessary to use the whole animal.

After removal from the animal, proteins begin to denature rapidly and so the tissue must be used immediately or stored deep frozen. Storage at -190°C retains 90% of initial activity indefinitely and at -40°C most tissues will remain usable for a year or more, but in a domestic deep freezer at -18°C degradation is relatively rapid and will produce zymograms with aberrant patterns.

Supporting media

The original material, filter paper, has now largely been replaced by a variety of gels. Cellulose acetate and agar have

large pore size and are used for separation by protein charge and for immunodiffusion. Starch and polyacrylamide have a pore size similar to the molecular size of many proteins so there is also a molecular sieving effect. The pore size of acrylamide gels is adjustable. Protein resolution depends on the pH and ionic strength of the buffer, the pore size of the gel and the current applied. Buffer pH is usually 8 to 9 ensuring that all proteins migrate to the anode. Heat is produced in proportion to the product of volts X amperes so temperature must be controlled in order to prevent protein denaturation, either by having a water cooled base plate for the gel or by placing the entire apparatus in a refrigerator.

Types of electrophoresis

There are 5 main types of electrophoretic methods.

1. CONTINUOUS BUFFER ELECTROPHORESIS is the simplest and most commonly used method. The same buffer is used to provide electrical contact between the platinum electrodes and the support medium and to soak the gel. In the GRADIENT PORE method an acrylamide gel is prepared with a continuous gradient of pore size from one end of the gel to the other. Protein solutions are applied at the end of the gel where pore size is largest and allowed to migrate in an electrical field until they reach the point where pore size prevents further movement. Separation is purely on the basis of molecular size and the electrical charge is used only to induce movement.

2. In MULTIPHASIC ELECTROPHORESIS the electrode chambers contain a different buffer to that in the gel. The front at which the two buffers meet concentrates the different proteins so that they enter the main electrophoresis system as a very narrow zone. This enhances resolution.

3. SODIUM DODECYL SULPHATE (SDS) cleaves polymeric molecules into their constituent polypeptides and then binds them, imparting an overall negative charge masking individual variation. Electrophoretic separation is based solely on molecular weight.

4. Isoelectric Focusing uses a polyacrylamide gel with large pore size containing a mixture of polamino, polycarboxylic acids with different isoelectric points. These form a stable pH gradient along the gel in an electric field. Strong acid applied at the anode and strong base at the cathode contain and stabilise the gradient. Proteins migrate under the influence of their charge until they reach the point in the gel where the pH is equivalent to their isoelectric point (pI) and so their charge is neutralised. If they move away from this point they will develop a charge and so move back, thus becoming concentrated in a band on the gel. High resolutions are achieved permitting separation of proteins differing by only 0.01 pI.

5. Two Dimensional Electrophoresis: Additional resolution of protein mixtures can be achieved by using two different techniques in sequence and at right angles. The most usual method is to use isoelectric focusing in the first phase followed by SDS electrophoresis at right angles. The proteins are thus independently separated on the basis of their isoelectric point and molecular weight.

Band detection

Most protein are colourless so the separated bands can only be seen after staining. This can be done either with a general protein stain such as Amido Black or Coomassive Brilliant Blue or with specific stains which indicate particular proteins or enzymes.

It is possible to cut thick gels (6 mm) horizontally into 4 or 5 slices each of which can be stained for a different enzyme system. Alternatively proteins can be eluted from a band and subjected to peptide analysis to further identify proteins.

The various separation techniques will produce different degrees of resolution. With the same mixture containing 100 or more proteins cellulose acetate will produce 5 bands, starch gel 15, acrylamide 19 and isoelectric focusing 30 or more.

Two dimensional electrophoresis combining isoelectric focusing with SDS system can resolve 100 polypetides in human plasma.

Genetic basis of alloenzyme studies

In diploid, sexually reproducing animals each chromosome pair consists of homologous chromosomes one derived from each parent. Every gene (or locus) is composed of two parts, the **Alleles**, one on each of the homologous chromosomes. Each allele is composed of a section of DNA with the same or similar base sequences. When the alleles are codominant (which is the most common condition for protein producing loci) each allele forms half of the total amount of polypeptide. However, if one of the alleles contains a slightly different sequence of bases the locus will produce two polypeptides with the same function but differing from each other by minor amino acid substitution. A locus in a species is considered **Polymorphic** if the most common allele does not occur at least 99% of the time (some definitions say 95%).

In a species there may be a large number (30 or more) alleles at any locus. However, in the individual there are only two possibilities; the alleles at a locus are identical (**Homozygous**) or different (**Heterozygous**). If for example, in a population there are two possible alleles A and B, for a locus then three genotypes are possible. Two homozygotes AA and BB, and one heterozygote AB. Each homozygote will produce only one type of polypeptide but they will be different from each other, whereas the heterozygote will produce both polypeptides.

For example in the lobster *Homarus*, the glycolytic enzyme phosphoglucomutase (PGM) is present in three forms. All lobsters have PGM-2 which migrates as a single fast band, but PGM-1 occurs in three types in different individuals, either as single electrophoretic bands PGM-1^{103} and PGM-1^{100} (which migrates more slowly) or as two bands PGM-1^{100}_{103}. It is suggested that the single bands are products of homozygous alleles while the double band is produced by a codominant heterozygotic locus. Controlled crosses followed by progeny testing support this hypothesis. This illustrates that homozygous alleles

always produce proteins which migrate in a single band whereas heterozygotes produce at least two bands and as many as five depending on whether the enzyme has 1, 2, 3 or 4 polypeptides.

Figure 5 illustrates some simple zymograms produced by 2 or 3 codominant alleles producing either monomeric or multimeric proteins. Approximately half the proteins used in electrophoresis are dimeric. Null alleles are not uncommon in polyploids.

Analysis of zymograms

When individuals in a population exhibit variation in protein structure the zymograms will conform to those expected under simple models of single locus Mendelian inheritance with co-dominant expression.

Analysis of electrophoretic data

1. Calculation of allelic frequencies

The frequency of an allele is given by $\frac{2\,Ho + He}{2N}$

Ho = number of homozygotes at allele
He = number of heterozygotes at allele
N = number of individuals examined

'Standard error' of the frequency of allele P is estimated by

$$\sqrt{\frac{P\,(1-P)}{2N}}$$

2. Heterozygosity calculated by $H_1 = 1 - \sum X_1^2$

X_1 = frequency of the 1^{th} allele at a locus

Mean heterozygosity per locus $\bar{H}_1 = \frac{\sum H_1}{N}$

$\lesssim H_1$ = sum of over all loci examined

N = total number of loci examined

The variance of H_1 for r loci is $V = \dfrac{\lesssim (H_m - \bar{H}_1)^2}{r\,(r-1)}$

H_m = Heterozygosity at the m^{th} locus

Standard error of $H_1 = \sqrt{V}$

If the population is in Hardy-Weinberg Equilibrium the calculated and observed heterozygosities will not be significantly different.

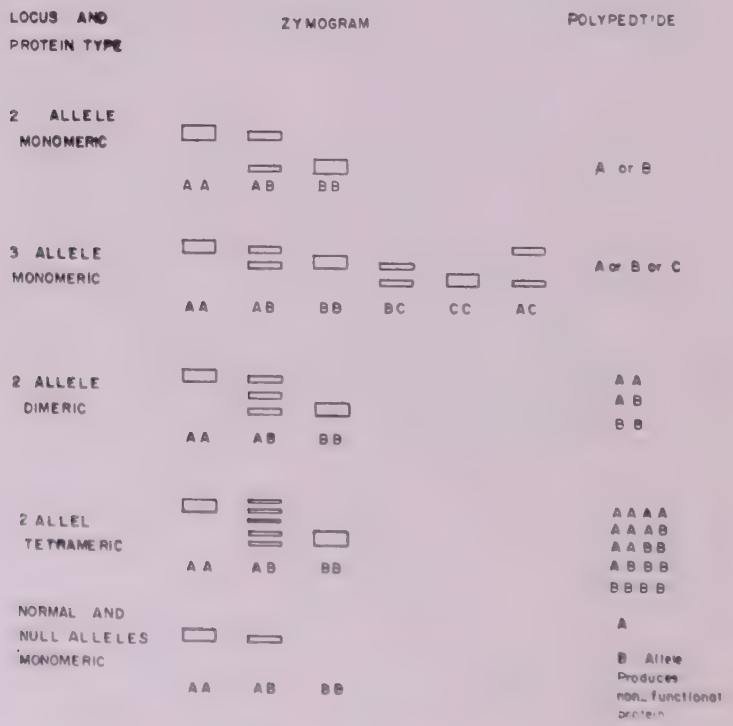

Fig. 5. Zymograms.

The mean heterozygosity per individual is the mean of the proportion of loci at which each individual is heterozygous,

summed over all individuals. Thus if, on an average, an individual is heterozygeons at 12 of the 40 loci examined, its mean heterozygosity is 0.3.

Limitations

Electrophoresis is not a perfect method for detecting genetic variations since it examines only the structural genes which comprise approximately 1% of the genome. In addition, because most amino acids are electrically neutral and frequently insignificantly different in molecular weight, probably less than 25% of all amino acid substitutions can be detected by electrophoresis. However, variation in technique and sequential use of different methods should permit the detection of a substantially higher proportion. Techniques for examing non-structural genes are also being developed.

The main effect of this inability to detect variation is that heterozygosity is likely to be markedly underestimated and the apparent similarity between populations overestimated. So electrophoresis cannot demonstrate similarities but only differences. It is very inefficient even in this task.

To date there has been limited success in attempts to couple electrophoretically identified protein variability with economically important characters although the positive correlation between degree of heterozygosity and overall performance has been demonstrated in a variety of fish and molluscs.

Despite these limitations, the examination of protein variation permits some evaluation of the amount of genetic variation in natural populations and the subsequent detection of genetic changes following domestication.

Practical applications

In modern aquacultural operations great emphasis is placed on selective breeding, preservation of genetic variation, inbreeding

and crossbreeding, hybridization and genetic engineering. All these practices require genetic characterisation of the stocks under investigation and analysis of results uninfluenced, as far as is possible, by environment. Biochemical genetics is the only practical method of obtaining a sufficiently detailed characterisation of a statistically acceptable number of individuals within a population.

For the development of inbreeding and cross breeding programmes biochemical genetics permits the evaluation of the degree of homozygosity and the genetic similarity of populations making designed crossings more likely to be productive.

These techniques also make it possible to monitor genetic changes in cultured populations thus permitting the detection and correction of unitentional inbreeding and gene loss.

The breeding of specific rare alleles into populations to serve as genetic tags is likely to be a very valuable development. It will permit the evaluation of the performance of different stocks of animals in the same environment and can be used with animals where initial size or life styles precludes normal tagging methods. Evaluation of the performance of hatchery reared animals stocked in natural conditions is possible following allozyme tagging.

With hybrid animals electrophoresis allows the detection of the relative contribution of maternal and paternal genomes. In addition the characterisation of species makes it possible to predict, to a certain extent the outcome of hybridization.

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7

SEX CONTROL AND CHROMOSOMAL ENGINEERING

Monosexuality

Although in fish, as in other animals, sex is genetically determined, many fish are not clearly differentiated into either sex at hatching. If the fry are fed a diet containing sex steroids during the time that their gonad is differentiating, they will develop as male or female depending on the nature of the hormone used. Thus stocks which consist entirely of males or entirely of females can be produced.

Direct feminization has been successful with some species but the method has not proven so reliable with others. Direct masculinization is frequently easier to achieve. A disadvantage of the technique is that all fish require treatment and fish destined for human consumption will have been fed steroid hormones. Although the hormone dose is minute and all residues are eliminated well before marketing the knowledge of the treatment may provoke consumer resistance.

To counter this difficulty it is possible to develop an alternative method of producing entirely female fish which avoids hormone treatment of those which will be eaten. Only the relatively small number of male broodstock which are required, need to be treated

Paradoxically the procedure is started by feeding larval fish with food containing a male sex steroid which converts the genetically female fry into functional males although they retain the genetic constitution of a female. When these fish mature, their milt is used to fertilise the eggs from normal, untreated females. Succes with this method depends on the sex determining mechanism of the animal being the same as in humans with the female producing eggs which are all of the X type and the male producing two different types of sperm containing either X or Y chromosomes. If an egg is fertilized by X sperm it develops into a female but if it is fertilized by Y sperm a male develops (Fig. 6 a). In this method a special type of male is created which can only produce the X type of sperm so that all the eggs it fertilises will become females (Fig. 6 b).

Steroid reversal for backcrossing, in species with female heterogamety or other sex determining systems is often less useful unless entirely male populations are required.

Sterilization

The alternative to the production of monosex stock is to suppress sexual development completely. Several methods are available to induce sterility including surgical castration, autoimmune gonad rejection, the administration of high doses of sex steroids or chromosomal manipulations.

Surgical castration is impractical for some organisms and is unlikely to be an economic proposition for any farmed animal. Autoimmune castration involves the injection of minced gonad into immature fish to encourage the production of antibodies which will destroy the fish's own gonad as it starts to develop. Some initial success with this technique has been claimed for salmonids but the results are unconvincing and in other trials with marine flatfish the gonads of treated fish developed normally.

If young fish with relatively undifferentiated gonads are fed with high doses of sex steroids all gonad development is suppressed.

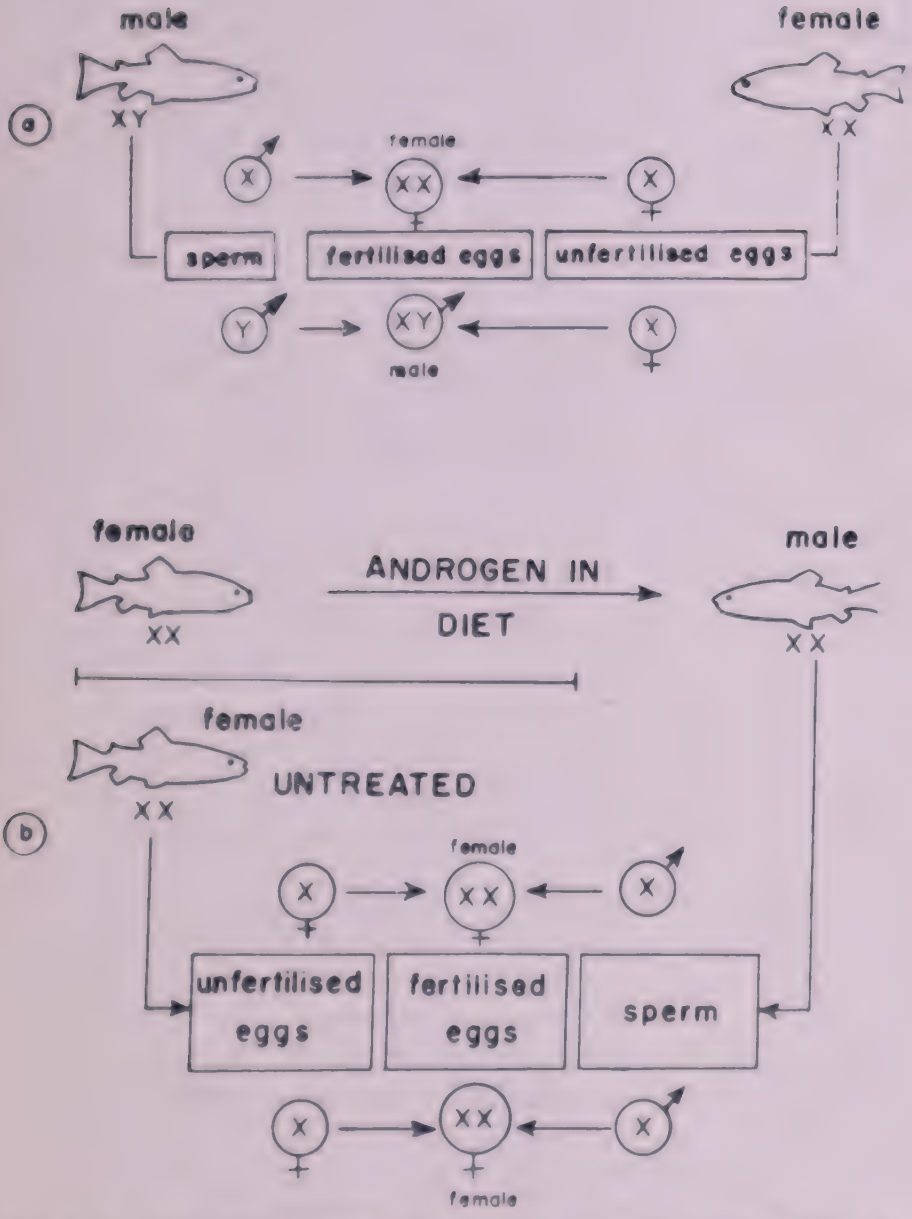

Fig 6. a. Sex determination in fish with male heterogamety and b. Production of all female stocks by masculinization and back crossing.

Note. All female populations produced have received no steroid treatment. Only the male broodstocks have received small doses of androgen.

However, the concentration of hormone and the duration of treatment must be carefully controlled to avoid depressed growth and high mortality and to ensure effective sterilisation. A safe and reliable system for some flatfish has been developed but the effects on other species are much more variable and it is probably not a commercially practical method.

Chromosomal Manipulation

Chromosomal manipulation, however, does appear to be a suitable technique for inducing sterility in some aquatic organisms. The chromosome number of the organism is increased by subjecting the egg to a pressure or temperature shock shortly after fertilization. This prevents the normal expulsion of one set of maternal chromosomes so that after fusion of the chromosomes from the sperm the developing embryo contains three sets of chromosomes instead of the normal two sets. The triploid animals which develop are often virtually identical to normal diploids except in the gonads where the extra set of chromosomes interferes with gamete formation. In the triploid marine flatfish gonad development is abnormal in both sexes although the males produce some spermatozoa. Although male triploid salmonids develop a normal sized testis the growth of the ovary in the female triploid is completely suppressed.

Although the complications of sexual maturation are avoided, it is possible that the growth rate of sterile fish will initially be slower than that of the diploids because the low doses of sex steroids secreted by the immature gonads of normal fish may be necessary to obtain the best growth rate. However, when the diploids mature their growth rate will be reduced while that of the tripoids is unaffected.

Another chromosomal engineering technique which is potentially valuable for fish farming is that of gynogenesis. Gynogenomes are animals derived entirely from maternal chromosomes. Milt is irradiated by ultraviolet or gamma rays which destroy the genetic material but leave the sperm able to swim into the egg and

initiate development. The egg chromosomes are induced to duplicate by pressure or temperature shock so that the animal has the normal diploid number of chromosomes but they all originate from the mother (Fig. 7).

In normal development, second meiotic division in the egg occurs around the time of ovulation. The egg receives a haploid set (n) of chromosomes as the male pronucleus from the spermatozoan which fuses with the haploid pronucleus of the egg. The other haploid set of maternal chromosomes are extruded as the second polar body. The diploid (2n) blastodisc containing maternal and paternal genetic material commences mitotic division and forms a diploid embryo.

In gynogenesis the irradiated spermatozoan enters the micropyle of the egg and induces development, but then degenerates without making genetic contribution. The resulting haploid embryo which contains only maternal chromosomes is grossly abnormal and rarely survives to hatching.

Gynogenetic diploids can be produced in two ways.

1. By subjecting the egg to a thermal, pressure or chemical shock shortly after fertilization thus suppressing extrusion of the second polar body and creating a diploid embryo derived entirely from maternal chromosomes. This will be 50 to 100% inbred (homozygous) depending on the extent of chromosomal crossingover during early meiosis.

2 By shocking the egg just before the first division of the blastodisc, inhibiting mitosis and inducing the recovery of diploidy. This embryo will be 100% homozygous. In androgenesis an egg in which the genetic material has been destroyed by irradiation, chemicals or ageing is fertilized by a normal spermatozoan. Diploidy is restored by physical or chemical treatment of the egg just before the first mitotic division producing a diploid embryo derived entirely from paternal chromosomes. This embryo will be 100% homozygous. In polyploidy, physical or chemical shock applied

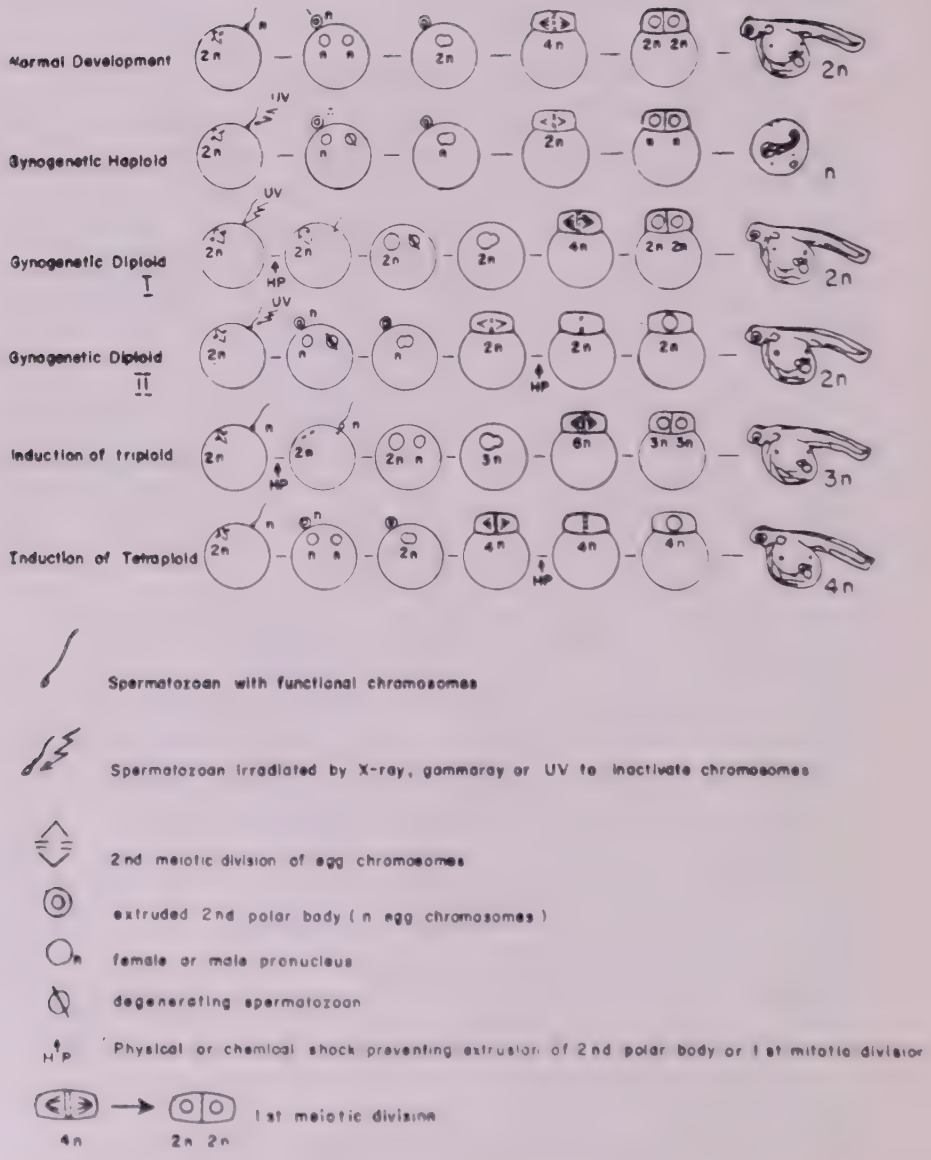
Normal Development
Gynogenetic Haploid
Gynogenetic Diploid I
Gynogenetic Diploid II
Induction of triploid
Induction of Tetraploid
UV
HP
Spermatozoan with functional chromosomes
Spermatozoan irradiated by X-ray, gammaray or UV to inactivate chromosomes
2nd meiotic division of egg chromosomes
extruded 2nd polar body (n egg chromosomes)
female or male pronucleus
degenerating spermatozoan
Physical or chemical shock preventing extrusion of 2nd polar body or 1st mitotic division
1st meiotic division
4n
2n 2n

Fig. 7. Manipulation of chromosome sets in fish.

to a normally fertilized egg induces triploidy if it supresses extrusion of the second polar body and tetraploidy if it inhibits the 1st mitotic division. Repeated shocking before each mitotic division can induce multiple ploidy.

The sex ratio of the offspring after gynogenesis provides information about the sex determining system in the species. Because the female is homogametic, gynogenetic trout which have been produced are all female. However, because of relatively poor survivals following shocking this would not be a practical technique for producing monosex populations but it does provide a useful tool for producing inbred lines for subsequent F1 hybridization. Gynogenesis produces the same degree of inbreeding as 6 generations of sib mating which in salmonids represents a saving of at least 18 years for each generation of gynogenesis. Second generation gynogenetic rainbow trout have been produced and following masculinization of some by steroid treatment, crossing will be performed to form the basis of inbred stains.

Androgenesis, the development of organisms entirely from the paternal chromosomes, has not received much attention in aquaculture. However, it does have the potential of producing all male offspring in species with male homogamety.

Gynogenesis and androgenesis followed by sex reversal with steroids can be used to create homogametic XX and YY males for producing outbred monosex populations.

PART 2

8

CYTOGENETICS

Introduction

Of the vast range of organisms of potential value for aquaculture details of chromosomal constitution and morphology are known for less than 1%.

Obtaining chromosome spreads of sufficiently good quality for detailed examination is a limiting factor in cytogenetics. Until the advent of new techniques in mammalian cytology in the 1960's only histological sectioning techniques provided reproducible results. The three major innovations which facilitated the preparation of adequate numbers of well spread chromosomes were

i. Pre-treatment with mitotic inhibitors such as colchicine which caused the accumulation of metaphase spreads;

ii. Exposure of cells to hypotonic solutions inducing swelling and thus spreading the chromosomes, and

iii. Air drying of tissues on to microscope slides producing well spread chromosome sets in one focal plane.

Theoretically chromosome preparations can be prepared from any tissue which is actively dividing and methods are available for

most tissues. However, in aquatic organisms preparations from embryos and from the soft organs (spleen, kidney, liver) of juveniles and adults have been most frequently used because the techniques are rapid and relatively inexpensive. Equally good sources are the epithelial cells of gills, fins and scales, particularly if the animals are pretreated with a mitotic inhibitor. Gill preparations have been frequently used in molluscs.

Testes can be used for both meiotic as well as mitotic chromosome preparations but usually they can only be sampled during active spermatogonial division. Testes preparations have been particularly useful in crustacea. Care must be taken with the use of mitotic inhibitors on testes since over dosing is likely to result in polyploidy.

Preparation of chromosome spreads from cell cultures has been particularly successful in mammalian cytology. Many techniques have been applied to fish and that with most potential appears to be leucocyte culture. However, although these methods can produce exceptionally good results they are difficult, time consuming and expensive.

For reviews of cytogenetics and chromosome techniques readers are referred to Denton (1973), Gold (1979) and Schulz-Schaeffer (1980).

A selection of techniques for chromosome preparation and an introduction to methods for karyotyping are included in this handbook.

Use of Karyology

The karyotype may be used to identify a species, hybrids within species or even strains of a species, in a similar manner to the use of biochemical genetic markers. However, because of present inadequate cytological techniques and chromosomal polymorphisms which may occur even within an individual animal

this approach must be used with caution. Cytological identification of strains and hybrids of aquatic organisms are few.

However, as a first step in a genetic programme it is valuable to establish the karyotype and its normal variability, of all organisms of potential use for aquaculture. This will frequently indicate in which organisms there is chromosomal compatability and hence the possibility of hybridization.

The structure of the sex chromosomes will sometimes indicate which type of sex determining mechanism operates in an organism and which of the sexes is heterogametic. This is obviously of value in the design of procedures for artificial sex control (see Chapter on Sex control and Chromosomal Engineering). The efficiency of chromosomal engineering programmes in producing haploid, parthenogenetic and polyploid organisms can be most accurately assessed by karyotyping.

Failures in embryonic development do not result only from environmental problems, they are frequently caused by chromosomal abnormalities. These may arise because of delayed fertilization, aging of eggs or sperm, nutritional inadequacies in the broodstock, or various types of pollution. Persistent failure in the embryonic development of cultured organisms warrants examination of karyotypes as well culture techniques.

Chromosome morphology can also be used as an indicator of damage caused by pollution of the environment with chemical mutagens or radiation. However, because of the possibility of chromosomal abnormalities induced by preparation and staining it is essential to include control organisms from unpolluted areas.

Chromosome Techniques

The techniques described in this handbook have been developed for fish and crustacea from the Northern Temperate Zone and it will usually be necessary to modify the methods to obtain optimal results.

All stages of the techniques can be modified to give improved results for the particular species and tissue under examination. Experiment to improve the technique must be conducted in a methodical way with appropriate controls. See Blaxhall (1983) *J. Fish. Biol.*, **22** : 61-76.

By comparison with mammals, on which the majority of cytological studies have been performed, the chromosomes of aquatic organisms are often small and numerous. Karyotyping has been inhibited by the difficulty of obtaining satisfactory numbers of well spread metaphases. The techniques used for fish chromosome studies include histological sectioning, electron microscopy, air drying cell suspensions, squashing embryonic or adult tissues or the culture of leucocytes or fibroblasts (reviewed by Roberts, 1967; Denton, 1973; Blaxhall, 1975; Gold, 1979). Although the solid tissue suspension and squash techniques do not provide as many and as well spread metaphases as leucocyte or fibroblast culture, they have the advantage of being cheaper, quicker and applicable to field situations. The semiculture technique (see method C) combines some of the advantages of both general methods.

There is always considerable individual variation with these methods so that under exactly the same conditions one sample will give hundreds of metaphase spreads while that from another animal of the same species will give none. In general, only 25% of samples will exhibit a usable number of mitotic figures and only 10 to 20% of these will be suitable for karyotyping. Shortening, clumping and overlay of chromosomes or cell rupture will make many spreads unusable.

A

LEUCOCYTE CULTURE

1. Based on Hartley and Horne 1983, *J. Fish. Biol.*, **22**: 77-82.

   (Note: During all procedures attempt to maintain aseptic conditions)

   a) Collect up to 2 ml of blood with a heparinized syringe.

   b) Place blood in centrifuge tube and centrifuge at slow speed (approx. 100 rpm) for 10 minutes.

   c) Suck off plasma and buffy layer of cells from top of red cells. Retain.

   d) Centrifuge twice more at high speed (800 rpm) and pool plasma, buffy layer fractions. Approx. 1 ml of leucocyte-rich plasma will be obtained from 2 ml of whole blood. Combine plasma from (i) immature and (ii) mature fish.

   e) Innoculate 0.25 ml plasma into 5 ml of medium 199 with antibiotic antimycotic, foetal calf serum and phytohaemagglutinin (formula appended).

f) Incubate for 5 days at 20°C.

g) Add colchicine to give 5 $\mu g\ ml^{-1}$. Shake gently and leave for 4 hours at 20°C.

h) Centrifuge at 1000 rpm for 10 minutes. Pipette off supernatant and discard.

i) Shake up cells in 5 ml of 0.075 M KCl for 10 minutes From this stage treat cells very gently - they are fragile.

j) Centrifuge at low speed for 5 minutes. Pipette off hypotonic solution.

k) Add 2 ml of freshly prepared methanol: glacial acetic acid mixture (3:1) and suspend pellet by stirring. Leave 15 minutes. Centrifuge at low speed for 10 minutes.

l) Replace fixative twice at 15 minute intervals.

m) Pipette off fixative leaving a few drops in which to resuspend the cells.

n) Drop 1 to 2 drops of suspension onto a clean, grease-free slide held at 60°. Air dry at room temperature. The dried spreads can be stored in a covered box in a refrigerator for upto 4 months.

o) Stain with 5% Giemsa in 0.1 M phosphate buffer at pH 6.8 for 10 minutes.

p) Coverslip to examine wet mount or;

q) Rinse off excess stain, take through absolute ethanol, absolute ethanol: xylene (1:1), then pure xylene twice, with about 2 minutes in each treatment. Mount and coverslip.

Variables

a) At stage (g) substitute colchicine with COLCEMID (Demecolcine) to give a final concentration of 0.1 to 0.3 μg ml $^{-1}$ Incubate for 4 hours at 20°C.

b) Use culture medium containing either PHA (M) or PHA (P) with the same leucocyte samples.

c) At stage (e) reduce innoculum. Use 0.1 ml leucocyte-rich plasma.

d) Reduce incubation period to 3 days.

Culture medium

a) Make up 1 litre medium 199 (GIBCO) with sterile distilled water.

b) Add 0.65g $NaHCO_3$

c) Into 50 ml of medium with bicarbonate, add 0.1 ml of antibiotic/antimycotic solution (GIBCO).

d) Add 7.5 ml Foetal calf serum.

e) Add 3 ml of PHA (M) solution (GIBCO) or PHA (P) (Wellcome).

f) Add 0.02 mg l^{-1} cholesterol.

g) For marine organisms increase NaCl content of medium to 0.2 molar.

Notes

a) Stages (e) and (f). The ideal container for culturing the lymphocytes is a 10 ml sterile plastic stoppered tube with a rounded base.

b) Other culture media are suitable, for example; McCOY's 5A (modified) medium. Eagles minimum essential medium Bicarbonate should be between 350 to 850 mg l^{-1}. Supplementation with 1–2% l–glutamine is required.

c) The addition of cholesterol at 0.02 mg l^{-1} may increase the % mitosis by upto 5 times.

d) PHA(M) concentrations of 12.5 to 50 µl ml^{-1} appear to be most effective.

e) PHA (P) gives more consistent results. Concentrations of 0.001 to 0.01 units ml^{-1} are effective.

f) The concentration of lymphocytes in the culture medium influences the incidence of mitosis. Optimum concentrations are usually 2 to 4 x 10^6 ml^{-3}. Concentration of lymphocytes can be calculated after counting with an improved Neubauer chamber.

g) Cultures may be stored for several days at 4°C before incubation commences.

h) Methanol/Acetic fixative must be made up immediately before use.

IB

CHROMOSOME PREPARATIONS FROM SOLID TISSUES

1. Based on Gold 1974, *Prog. Fish. Cult.*, **36** (3): 169-171. and Kligerman and Bloom 1977, *J. Fish. Res. Bd. Canada*, **34:** 266-269.

   a) Allow fish to swim in 0.005-0.01% colchicine solutions for 6-18 hours if too small to inject. But, in preference, inject intraperitoneally with colcemid (Demecolcine) or colchicine at 25 $\mu g\ g^{-1}$ body weight in 0.85% sterile NaCl solution and leave for 3 to 5 hours.

   b) Kill fish and dissect out testis, kidney or spleen. If fish have been swimming in colchicine solution fin margins, scales and gills may also be used. Cut tissues into pieces about 1 mm cubes.

   c) Place tissue pieces in 2 ml of 0.4% KCl solution for 20 to 50 minutes. If possible monitor swelling of cells under a microscope.

   d) Pipette off hypotonic solution and fix tissues in 3 changes for at least 30 minutes each, of 3:1 methanol: glacial acetic acid. Make up the fixative immediately before use.

e) Remove pieces of tissue, blot lightly on filter paper to remove excess fixative and place in the well of a cavity slide in 2–3 drops of 50% acetic acid.

f) Mince tissue with scalpel or fine dissecting needle for a minute to make a cell suspension. Replace remaining tissue fragments in fixative.

g) Using a fine pasteur pipette or microhaematocrit capillary tube with a rubber bulb draw up a drop of the suspension and expel onto an acid cleaned slide heated to 40–50°C.

h) Quickly suck drop back into pipette to leave a ring of cells and repeat a few times to leave a number of concentric rings.

i) Repeat (g) and (h) producing 3 to 5 sets of rings on each slide. The dried slides can be stored in a covered box in a refrigerator for upto 4 months.

j) Stain in 5% Giemsa in 0.1 M phosphate buffer at pH 6.8 for 10 minutes. Wash of excess stain in distilled water.

k) Cover slip to examine wet mount; or

l) Rinse off excess stain in distilled water and dehydrate through absolute ethanol, absolute ethanol: xylene mix, then two changes of xylene with about 2 minutes in each treatment. Mount and coverslip.

m) The metaphases can be easily located as they are concentrated on the periphery of the rings.

Variables

a) The tissue mincing at stages (e) and (f) can be replaced by rapid homogenizing in 50% acetic acid.

b) Tissue suspensions can be dropped from 30 cm onto hot slides inclined at 60°. This may improve spreading.

Notes

a) Raising the slide temperature increases the number of dried cells and improves spreading but it also tends to scatter chromosomes and interfere with their morphology.

b) Increased time in acetic acid stages (e) and (f) can improve the spreading of chromosomes but can also burst the cells.

c) Higher acetic acid concentrations induce abnormal chromosome morphology and should be reduced to a level which still allows rapid dispersal of the tissue into a cell suspension.

d) This method is readily adaptable to a variety of band staining techniques and by the FPG method to reveal sister chromatid exchange.

e) This method is easily adaptable for use with bivalve molluscs. The molluscs are immersed in colchicine solution for 5-7 hours and then gill tissue is removed and treated as described for stage (b) onwards. Best results are obtained from spat or juveniles.

2. Based on Salemaa 1979, *Crustaceana*, **36** (3): 316-318 for Crustacea.

a) Inject 0.1% colchicine into the haemocoel and leave for 30 minutes.

b) Dissect out testis and cut into 1 mm pieces. Wash in 0.075 M KCl for 3 to 5 minutes.

c) Pipette off hypotonic solution and replace with 2 ml of methanol: acetic acid (3:1) fixative for 15 minutes.

d) Repeat with 2 x 1 hour changes of fixative.

e) Place a piece of tissue after blotting on filter paper into the well of a cavity slide in 2–3 drops of 50% acetic acid.

f) Mince tissue with scalpel or fine dissecting needle for a minute to make a cell suspension. Remove large fragments.

g) Using a fine pasteur pipette or a microhaematocrit capillary tube with a rubber bulb draw up a drop of the suspension and expel onto an acid cleaned slide heated to 40–50°C.

h) Quickly suck drop back into pipette to leave a ring of cells and repeat a few times to leave a number of concentric rings.

i) Repeat (g) and (h) producing 3 to 5 sets of rings on each slide. The dried slides can be stored in a covered box in a refrigerator for upto 4 months.

j) Treat slides for 15 to 40 seconds in 0.1% trypsin in 0.1 M phosphate buffer at pH 6.8.

k) Wash in 2 changes of buffer for 6 minutes each time.

l) Stain in 5% Giemsa in 0.1 M phosphate buffer at pH 6.8 for 10 minutes. Wash off excess stain in distilled water.

m) Cover slip to examine immediately; or

n) Rinse in distilled water and dehydrate through absolute ethanol, ethanol: xylene mix, then two changes of xylene with about 2 minutes in each. Mount and coverslip.

C

RAPID SEMI-CULTURE METHOD FOR MARINE ORGANISMS

1. Based on Barker 1972, *Copeia*, **2**: 365-368.

   a) Kill fish and dissect out spleen.

   b) Macerate in 2 ml of GIBCO medium 199 (see below). Wait for 2 minutes to allow fragments to settle.

   c) Pipette off about 0.5 ml of cell suspension and add to culture medium (see below).

   d) Incubate for 2 hours at 20° C.

   e) Centrifuge at 750 rpm for 5 minutes and discard supernatant.

   f) Resuspend pellet in 5 ml of 1% sodium citrate hypotonic solution and leave for 10 minutes.

   g) Centrifuge at 750 rpm for 5 minutes and discard supernatant.

   h) Gently resuspend cells in 5 ml of freshly prepared alcohol: acetic (3:1) mix and fix for 5 minutes.

i) Repeat step (g), add 5 ml of fixative and leave for 30 minutes. Repeat twice more.

j) Suspend pellet in 0.25 ml of 50% acetic acid.

k) Pipette a few drops of suspension onto hot slides in the ring method described previously.

l) Dry slides, stain, dehydrate and mount as previously described.

Notes

a) This method can also be used with kidney tissue or with whole blood using 0.25 ml of leucocyte rich plasma instead of tissue suspension at stage (c).

b) Injection of animals with PHA (M) at 0.2 ml g^{-1} 10 hours before killing may increase the incidence of mitosis.

Culture medium

a) Make up 1 litre medium 199 (GIBCO) with sterile distilled water.

b) Add 0.65 g $NaHCO_3$.

c) Add NaCl to adjust osmolarity to that of plasma.

d) Add GIBCO antibiotic/antimycotic solution at 0.02 ml per 10 ml.

e) Add 0.02 mg l^{-1} cholesterol. Use this medium for homogenizing tissues. For culture medium take 5 ml of medium, add 2 ml of foetal calf serum and 0.2 ml of colcemid solution (to give approx. 0.3 $\mu g\ ml^{-1}$).

D

SQUASH TECHNIQUES FOR EMBRYOS

1. a) Dissect out blastodisc at 16-64 cell stage from eggs which have been fixed in 3 x 10 minutes changes of alcochol : acetic acid (3:1).

   b) Stain cells in 5% Giemsa in 0.1 M phosphate buffer at pH 6.8 for 20 minutes.

   c) Transfer without rinsing to acid cleaned slide and squash gently under coverslip.

   d) Ring coverslip with wax, Kronig's cement, etc.

2. a) Place eggs containing well developed embryos in a 0.02% colchicine solution for 6 hours.

   b) Dissect out embryo from egg in 0.85% NaCl and cut off tail bud.

   c) Rinse in distilled water and transfer to distilled water for 15 minutes.

   d) Fix in ethanol : acetic acid (3:1) for 3 minutes and then rinse in distilled water.

   e) Transfer to cavity block and gently dissociate tail epithelium in 50% acetic acid.

f) Drop tissue suspension onto warm slide (50° C) and air dry.

g) Stain in 5% Giemsa for 10 minutes.

h) Rapidly rinse, dehydrate, clear and mount.

Notes

a) Bathing eggs in 0.01% colchicine solution for 4 hours before fixing may increase number of metaphase spreads, but can also induce chromosome clumping.

b) If yolk causes problems with staining the dissected blastodisc can be treated with acetone for 5 minutes, before rinsing in 2 changes of alcohol : acetic acid and staining.

c) During early stages of cell division upto 50% of cells will be in metaphase at any time. Cell size decreases as cell division proceeds and well spread chromosomes are not easily obtained.

E

SQUASH TECHNIQUE FOR MEIOTIC CHROMOSOMES OF TESTIS

a) Inject colchicine or colcemid at 25μg g^{-1} in 0.85% sterile NaCl into peritoneum or haemocoel. Leave 2-4 hours.

b) Dissect out testis, rinse in distilled water and cut into 1 mm pieces. Swell tissue pieces in 1% Sodium citrate solution for 10 minutes and then pipette off hypotonic solution.

c) Immerse swollen tissue in 2% aceto orcein in 50% acetic acid for 10 to 20 minutes.

d) Remove tissue and scrape a slurry of cells onto a microscope slide. Add a fresh drop of stain and apply a cover slip.

e) Remove air bubbles from beneath coverslip and then cover with filter paper. Squash tissues using gentle rolling pressure from thumb.

f) The chromosomes should be adequately flattened when no more stain is forced from beneath the coverslip. Too much pressure should be avoided.

g) Temporary mounts are made by ringing the coverslip with wax, lanolin, Kronig's cement, etc. These will last for several weeks particularly if they are stored in a refrigerator.

h) If permanent preparations are required the slides should be placed on a block of solid CO_2 for 10 minutes and then the coverslip can be flicked off with the tip of a scalpel blade. Most of the squashed material will remain on the slide. Both the slide and the coverslip can then be dehydrated and cleared in alcohol, xylene and then mounted.

Notes

a) Fixing the tissue in 3 x 30 minute changes of alcohol: acetic acid (3:1) between steps (b) and (c) may give better results.

b) Both haploid and diploid chromosome spreads will be seen in both bivalent and univalent forms. However, testes are particularly sensitive to colchicine and treatment in excess of 4 hours will induce a high frequency of polyploids.

c) During early stages of gametogenesis when cells are actively dividing colchicine treatment may be unnecessary.

F

ADVANCED STAINING METHODS

Mammalian cytologists have developed an array of staining techniques which result in differential banding of chromosomes and permit the identification of individual chromosome pairs. Banding may also indicate functional aspects of the chromosome (Hoehn, 1975). A bibliography of the literature on banding techniques may be found in Nilsson (1973) and Schulz-Schaeffer (1980). Although few of these have yet been applied to aquatic organisms they have considerable potential.

Giemsa banding following trypsinization

a) Air dried chromosome preparations are treated with trypsin solution (0.13% trypsin in 0.1 M phosphate buffer at pH 7.0) for 5 to 10 minutes at room temperature.

b) Rinse in 2 x 1 minute changes each of 70% ethanol and absolute ethanol. Air dry.

c) Stain in 1% Giemsa in pH 7.0 phosphate buffer.

d) Rinse twice in distilled water, dehydrate in alcohol, clear in xylene and mount.

Notes

a) The effect is an alternation of light and dark bands across the chromosomes. The banding patterns are similar in homologous chromosomes.

b) Duration of trypsinization must be adjusted to give the clearest banding patterns.

GENERAL POINTS OF METHODOLOGY

Chromosomes of aquatic organisms are frequently small and numerous and require a research quality microscope with oil immersion lens for adequate examination. Magnifications of about 2500 are necessary. A graduated mechanical stage is essential and a camera assembly very useful. Because of the variability of staining, phase contrast optics are extremely valuable, particularly for photomicrography. A projection facility aids in the preparation of karyotypes. To obtain maximum image clarity care should be taken with the adjustment of the microscope.

Prepared slides should be examined in a methodical manner and adequately spread metaphase plates recorded immediately or the stage coordinates noted for future detailed examination. Chromosomes can be measured directly through the microscope by use of a calibrated ocular scale. However, counting, measurement and detailed karyotyping is more easily accomplished on a photomicrograph or an image projected on to paper.

1. In order to obtain optimally spread and stained chromosomes it will be necessary to modify the listed techniques to suit the species and tissue under examination. Some suggestions have already been made at the end of each technique and they are summarised here.

    a) In all techniques the number of cells in mitosis and the morphology of the chromosomes are affected by the concentrations of mitogen (PHA), colchicine or colcemid, foetal calf serum and inorganic salts.

    b) In culture methods, reduction in the quantity of cell innoculum and in the duration of incubation may increase the number of cells in mitosis. For marine organisms the addition of cholesterol to the culture medium is indicated.

c) In order to obtain well spread chromosomes, but without loss due to cells bursting it is essential to carefully control the hypotonic treatment. Watching the cells swelling in hypotonic solution under a microscope will allow the optimum duration of treatment to be established. The additional period in hypotonic solution during centrifugation should not be ignored.

d) Culture media should be adjusted so that the osmolarity is equivalent to that of the plasma of the animal under investigation. This is facilitated by the use of an osmometer.

2. Colchicine solutions used for bathing animals prior to chromosome preparation should always be well aerated.

3. The microscope slides for chromosome preparation should be washed overnight (in tissue culture grade cleaning solution if possible), rinsed for 2 hours in tap water and then in 2 x 10 minute changes of distilled water.

4. Alternative staining methods can be used and may give superior results. Air dried smears or squashes may be stained in undiluted Giemsa for 6 minutes, transferred directly to 0.06M NH_4OH for 2 minutes, rinsed in tap water, destained for 10 seconds each in acetone and 1:1 acetone: xylene, and then cleared in xylene before mounting. Aceto orcein at 1–2% in 50% acetic acid for 10 to 20 minutes has been used successfully. The stain should be filtered just before use. Controlled destaining in 50% acetic acid is usually required. Staining of the chromosomes often becomes more intense overnight. Feulgen staining can be used when DNA identification is required. It is, however, too difficult and time consuming for routine preparations.

5. Heparinized blood samples can be stored in vacuum flask with wet ice for at least 24 hr before use. Whether other excised tissues can be stored in chilled culture media (without PHA and colchicine) for periods before use must be determined by experimentation.

Preparation of a Karyotype

A karyotype is the characterisation and analysis of a chromosome set within the nucleus of a given species. This includes the number, size and other morphological characteristics of the chromosomes. The convention for describing non-mammalian karyotypes has not been established but it is recommended that the mammalian convention be adopted.

Nomenclature

The majority of the chromosomes consist of 2 strands, the chromatids, joined together by a centromere. This sub unit provides the point of attachment of spindle fibres. The centromere position is the basis for chromosome classification. If it is approximately in the centre of the chromosome it is Median; if it is at the end of the chromosome it is Terminal; if it is located midway between the centre and the end of the chromosome it is Submedian; if it is located between the end of the chromosome and the submedian position it is Subterminal. The chromosome type is based on the centromere position and this is most

Table 1. *Nomenclature for designating chromosome type*

Centromere position	Arm ratio L/S	Chromosome type
Median	1.00 – 1.70	Metacentric
Submedian	1.71 – 3.00	Submetacentric
Subterminal	3.01 – 7.00	Subtelocentric
Terminal	7.01 →	Acrocentric

accurately designated by the calculation of arm length ratios. Values based on the length of the long arm divided by the length of the short arm are most commonly adopted (Table 1, Fig. 8).

The number of chromosomes in the cells of a species is relatively constant and comprises the diploid complement (2n) except in gametogenic tissue where it may be haploid (n). In the diploid complement each autosome, and usually also the sex chromosomes, have a homologue. Chromosome sets in aquatic organism range from 2n = 16 to 2n = 250. The chromosome length also varies from less than 1μm upto 30μm.

Fig. 8. Chromosome nomenclature.

Photokaryotype

This is the systematic arrangement of the full diploid complement of chromosomes from a photograph representing one well spread metaphase plate. The chromosomes are cut from the print and arranged by chromosome type and size. Ideally

chromosomes should be measured before they are arranged. Sex chromosomes or odd chromosomes should be grouped separately at the end. A line scale, species name, strain identification, sex and the diploid number should be added to the karyotype before it is photographed.

Eye karyotype

This is a drawing of the chromosome complement obtained by microscopic projection, camera lucida, or freehand record. The drawing is analysed by scoring chromosome types, but size ranging is not attempted. This is rapid and relatively accurate karyotyping method which does not require photomicrography.

Idiogram

This is an arrangement of the haploid complement of chromosomes according to the position of the centromere and in order of decreasing length. This may be done with photographic cut outs, but is usually presented diagrammatically with straight line chromosome representation. The idiogram is particularly useful when comparing the chromosome complements of two or more species.

Chromosome modifications

In some species occasional chromosomes will consistently exhibit morphological or staining irregularities. These are useful as marker chromosomes for specific karyotypes. Modifications may include one or two exceptionally large chromosomes – MEGACHROMOSOMES or small, dot-like MICROCHROMOSOMES. SUPERNUMERARY chromosomes are fragments appearing in addition to the normal chromosome set and are considered to be genetically inert. Secondary constrictions occur in some chromosomes in addition to that at the centromere. The piece of chromosome terminal to the secondary constriction is termed a SATELLITE. More detailed descriptions of chromosome morphology and classification may be found in Denton (1973), Gold (1979), Schulz-Schaeffer (1980) and Kirpichnikov (1981).

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9

TECHNIQUES FOR SEX CONTROL AND CHROMOSOME ENGINEERING

Sex Control by Steroid Administration

The application of either androgen or estrogen to the developing embryo or juvenile animal over-rides the endogenous sex determining mechanism and either directs it in a male or female direction or induces sterility by completely inhibiting gonadal development. Early studies were performed to investigate the sex determining mechanism but the economic importance of controlling sexual development in aquacultural organisms has recently encouraged a more practical approach. Monosex culture is of value in preventing breeding during trial introductions of non-indigenous species (*e.g.* grass carp) or when prolific breeding results in unacceptably small individuals (*e.g. Tilapia*), or when one sex has a higher productivity than the other under controlled culture conditions (*e.g.* salmonids). For steroid treatment to be effective it is essential that the exogenous steroid is administered during the entire period of natural gonadal differentiation. Frequently this occurs just after hatching but occasionally, it may be necessary to treat developing eggs or juveniles.

A variety of steroid compounds have been tested but the two most generally effective are the synthetic steroids 17∝ methyl

testosterone and 17 β estradiol. These are effective both in solution and when taken by mouth.

Estrogen bath treatments of eggs and yolk sac larvae have varied from 0.25 to 400 μgl^{-1} for either continuous immersion (2weeks) or 2 to 6 immersions of 2 hours each during the egg and fry stages. Usually a 5 to 25 μgl^{-1} bath for upto 2 weeks is effective. Concentrations administered in fry food range from 2 to 30 mg hormone per kg food and durations of treatment from 15 to 60 days. Feeding for 30 to 50 days at 5 mg kg^{-1} is usually sufficient. All estrogen treatments induce temporary depression of growth rate and higher doses result in increased mortality. Gonadal feminization in rainbow trout has also been induced by rearing them for 4 weeks after hatching in a solution of NN–dimethylformamide (0.1 ml l^{-1}) and 30 μgl^{-1} progesterone.

Approximately the same range of hormonal dosages have been used in attempted masculinization with 17$\propto$ methyl testosterone. In general lower doses are effective and frequently administration in food only, is required. Treatment at 1 to 3 mg kg^{-1} of food for 50 to 70 days is usually effective. Higher doses and longer treatment durations are likely to induce sterility. Low to moderate doses of androgen do not normally have a detrimental effect on growth or survival and in fact frequently act anabolically promoting improved growth.

With both hormones low doses and/or short treatment durations produce substantial percentages of hermaphrodite animals. If these exhibit simultaneous maturation they can be used for self fertilization. Synthetic steroids are oil soluble and must be dissolved in alcohol or acetone before admixture to water to make bathing solutions. Steroids can either be added to the oil component of diets during manufacture or can be dissolved in alcohol, mixed in with the finished diet and absorbed following evaporation of the alcohol. Since steroids oxidise in the presence of lipids, treated foods are best stored at – 15°C or lower and only sufficient thawed for each day's feeding. Only steroid-containing food should be fed during the sex inversion process. It is important

to supply food for as much of the day as possible in order to ensure that a suitable concentration of exogenous steroid is present in the animal for most of the 24 hour cycle. An alternative method of applying hormones is by intraperitoneal implants of silastic tubing filled with steroid. Doses around 5 mg are usually effective.

Prior to the design of sex control experiments using steroid hormones it is valuable to establish by histology the time and duration of sexual differentiation. Since the rate of gonad differentiation, feed rate and steroid absorption are all directly related to temperature, adjustments to treatment durations may be required. Treatment for 300 to 800 C × days (mean daily temperature °C x number of days) is usually indicated.

For evaluation of the effects of treatment, histological assessment of juveniles provides an early result. However, it is essential to retain some of the animals to maturity to ensure that their genetic sex does not reassert itself.

Sex inversion may influence the morphology of the reproductive ducts preventing normal gamete release. Under these circumstances it will be necessary to remove the gametes surgically if they are to be used in a back crossing programme to produce monosex populations.

Sex Control by chromosomal Engineering

Gynogenesis and androgenesis can result in the production of monosex populations if the sex determining system of the animal is appropriate. Gynogenetic salmonids are entirely female because the female is homogametic whereas in plaice, a marine flatfish, gynogenomes include both sexes. In species where the male is homogametic, androgenesis provides a potential method for producing monosex offspring. However, in both cases the treatments required to inactivate the chromosomes of the spermatozoa or eggs and to induce diploidy result in a significant mortality rendering those techniques unsuitable for routine production of monosex populations. Nevertheless they are useful for rapidly producing

inbred lines and in association with steroid induced sex inversion permit the production of monosex F1 hybrids.

Inactivation of spermatozoan chromosomes is most easily achieved by irradiation. Treatment with X-rays or gamma rays from Co^{60} or Cs^{137} sources act by inducing chromosome breaks. Because of this, increasing durations of irradiation produce the classic 'Hertwig' effect where increasing radiation dose results in a dramatic decrease in embryonic survival followed by an increase in early survival at even higher doses. This effect is attributed to total destruction of the parental chromosomes at high doses resulting in haploid embryos which survive longer than the diploid embryos expressing dominant lethal mutations induced by lower radiation levels. However, even with high level treatment residual paternal characteristics or chromosome fragments may be found in the gynogenetic embryos. Effective irradiation doses have been of the order of 120 to 150 Krad (1 rad = 1 Joule = 100 ergs g^{-1}) ideally administered over a period not exceeding one hour. Subsequent survival of the spermatozoa is facilitated by diluting the milt with extender solution and packing in ice during irradiation. Fertilization rates are reduced with irradiated milt so at least double the usual quantity is required to give acceptable results.

Ultraviolet irradiation is easier to work with and less dangerous than gamma or X-ray sources. In addition it inactivates the chromosomes by inducing thymine dimers which cause clumping and so there is limited possibility of chromosome fragments interfering with gynogenetic development of the embryo. However, the effect can be reversed by a photoreactivation process which may occur in visible light, so exposure of irradiated milt or even of transparent eggs fertilized with irradiated milt should be avoided until embryonic development has started. UV light has poor penetrating power compared to gamma and X-rays so it is important to hold the milt in a thin transparent layer which is shaken or stirred during treatment. Dilution with extender and maintenance of low temperature enhances spermatozoan survival. UV irradiation doses of 10^3 ergs mm^{-2} or of 5 $mWcm^{-2}$ for 20 minutes are

generally effective. Total dose to ensure complete inactivation of sperm DNA has been calculated as 75 $mWcm^{-2}$. Ideally, in experiments for calculating the optimum irradiation dose a UV meter calibrated to the UV source should be used.

Denaturation of egg chromosomes to induce androgenesis has been reported with gamma irradiation and may be possible with UV irradiation. However, damage to cytoplasmic components may also result creating embryonic abnormalities. A low frequency of androgenomes can be produced by fertilizing aged eggs or by physically (temperature or pressure) shocking eggs shortly after fertilization.

Chemicals which induce mutagenesis can also inactivate the chromosomes in the sperm for gynogenesis and may be effective for androgenesis. Toluidine blue, ethylene urea and dimethyl sulphate have been used successfully, but concentrations and durations of treatment would need to be identified for each species.

Production of diploid parthenogenetic individuals and triploid or polyploid animals requires either retention of the second polar body of the egg or supression of the first mitotic division in the fertilized egg. Either of these events may occur spontaneously at a very low rate but for practical purposes they must be induced by temperature, pressure or chemical treatment of the egg at an appropriate time after fertilization. The timing and duration of treatments need to be determined for each species, but in general to suppress second polar body extrusion the shock should be applied shortly after fertilization and to block the first mitotic division it should be applied shortly before the first cleavage of the blastodisc appears.

Temperature shocks are easy to apply and offer the possibility of commercial application. Temperatures just above or below lethal limits are usually effective. However, it will be necessary to establish a compromise treatment which induces the higher level of diploidy or polyploidy consistent with an acceptable survival rate.

Hydrostatic pressures of about 700 atmospheres have been effective and may be less damaging to the egg.

A variety of chemical treatments have been successfully employed including cytochalasin B at 10 μg ml^{-1} in solution bathing the eggs, colchicine at 0.01% and polyethylene glycol. However, since they are less adaptable to mass production, unless they confer significant improvements in effect or survival they are unlikely to be the method of choice. Chemical treatments frequently produce polyploid mosaics.

Haploids are easily distinguishable from diploids by chromosome counts or external morphology. Haploid embryos have short twisted bodies, vacuolated body cavities, small eyes and poor vascularization of the yolk sac.

In diploid gynogenomes it is important to be sure that there is no paternal genetic contribution. This can be checked by using males from a different species or males with particular colour, morphological or biochemical characteristics. Biochemical genetic polymorphisms can also be used to calculate the degree of homozygosity of a diploid gynogenome thus indicating whether it resulted from suppression of second polar body extrusion or first mitotic division. In the latter case progeny would be 100% homozygous.

Polyploids can be identified most accurately by karyotyping or measurement of DNA content. Measurement of nuclear volume or area in erythrocytes, cartilage cells or retinal neurons can also be realiable as long as it is first calibrated against chromosome counts. The formula $a^2.b/1.91$, where a = minor axis and b = major axis of the nucleus, provides a reasonable estimate of nuclear volume although in many cases the long axis alone is sufficient to distinguish diploids and triploids. When sterility is associated with polyploidy, examination of gonad squashes may be sufficient.

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10

TECHNIQUES FOR THE MANIPULATION AND CRYOPRESERVATION OF GAMETES

The ability to freeze the gametes of aquatic organisms and to store them for long periods without deterioration would be of considerable value in the genetic improvement of aquaculture. It would facilitate the crossing of strains or species which are geographically seperated or spawn at different times and it would allow the self-fertilization of sequential hermaphrodites. In addition it would permit the preservation of gene pools, both for their intrinsic value and in order to monitor the progress of selection

Long term storage of aquatic eggs and embryos has met with little success, but because there are now reliable techniques for freezing and storing mammalian embryos it should not prove impossible. Initially, however, it may be more profitable to concentrate on the cryopreservation of spermatozoa since their small size and relatively simple structure make them more resistant to the procedures employed. Techniques have been developed for a number of aquatic species and in general the spermatozoa of marine organisms survive freezing better than those from fresh water animals.

Lannan (1971) developed a method for the oyster *Crassostrea gigas* and although the fertilization rate and larval survival were

low it did permit subsequent self-fertilization. Methods for the freezing of marine fish spermatozoa have been reported (Mounib, 1968; Pullin, 1972) although they will undoubtedly need to be modified for other species. It has not yet been possible to establish a reliable cryopreservation technique for salmonid milt but the review of Scott and Baynes (1980) and the paper of Baynes *et al.* (1981) are recommended as providing details of the range of parameters which should be investigated.

Manipulation of gametes

As soon as eggs are ovulated they start to deteriorate and the rate of deterioration is directly proportional to temperature. Much of the unsatisfactory fertilization, hatching and larvel survival in aquaculture is due to delays between ovulation and fertilization, particularly in artificial fertilizations. For *in vitro* fertilization eggs should be collected as soon as possible after natural or induced ovulation and if they are not fertilized immediately they should be cooled and stored at 2 – 4° C. They should always be collected in ovarian fluid and not in water and should be stored not more than 2 or 3 layers deep. They can be kept in this way for 24 to 36 hours. Milt should be stripped into dry tubes without contamination by urine or faeces and if not used immediately should be cooled and stored at 2–4° C, in a layer not more than 5 mm deep. Gassing with oxygen promotes survival. Chilled milt will often survive several days storage. Sperm may be diluted with an extender with a chemical composition similar to that of seminal plasma. As a preliminary to cryopreservation trials it is valuable to investigate the chemical composition of the milt (pH, Na^+, K^+, Ca^{++}, Mg^{++}, osmolarity, etc.), the sperm count, spermatocrit, milt volume and motility. It should be noted that sperm motility is not necessarily synonymous with the ability to fertilize.

Fertilization

As a general rule artificial fertilization, is most successful when performed 'dry' *i.e.* the eggs and milt are mixed before water is added. Union of the gametes normally occurs within a few

seconds. 'Wet' fertilization using a buffered saline solution may give better fertilization rates than the dry method (perhaps because it prevents blocking of the micropyle with yolk from broken eggs) and a more economical use of milt.

Cryopreservation of milt

Before freezing it is necessary to dilute the milt with an extender solution. This is a mixture of salts at an appropriate pH which helps to maintain the viability of the spermatozoa during refrigeration and does not activate them. For most animals sperm activation is a once-only action and the cell cannot be reactivated. The extender solution also contains a cryoprotectant which is thought to bind electrolytes during the freezing process, thus preventing them forming lethal concentrations. They also lower the freezing point of the intracellular fluids to around –45 °C and reduce ice crystal formation. Glycerol, ethylene glycol and propylene glycol have been used as cryoprotectants for fish sperm, but the most widely effective is dimethyl sulphoxide (DMSO). The rates of freezing and thawing affect the subsequent viability of the spermatozoa and will probably have to be established independently for each species. However, freezing rates of 30 to 160 °C per minute have generally been successful. Thawing has usually been accomplished by dropping the frozen milt directly onto the eggs to be fertilized or thawing to a slush immediately before adding to the eggs. Frozen milt cannot be thawed and then stored before it is used for fertilization. The cryopreservation method which follows is one which has been developed for marine fish milt at the Fisheries Laboratory, Lowestoft, U.K. It will probably require modification for other species.

Cryopreservation method for marine fish milt (Fig. 9)

a) Freshly stripped milt is mixed in the proportion 1:3 with extender solution (see formula below) held at the same temperature as the milt. If possible the mixture should be frozen immediately but it can be stored for up to 15 minutes on ice if necessary.

b) Freeze 200 μl aliquots of extended milt on a block of solid CO_2. Once frozen, handle only with cooled forceps and do not allow to warm up. Drill 0.5 cm diameter depressions into the top surface of the CO_2 block and using a repeater pipette fill each with 200 μl of milt mixture. Store frozen pellets in freezer vials in liquid nitrogen.

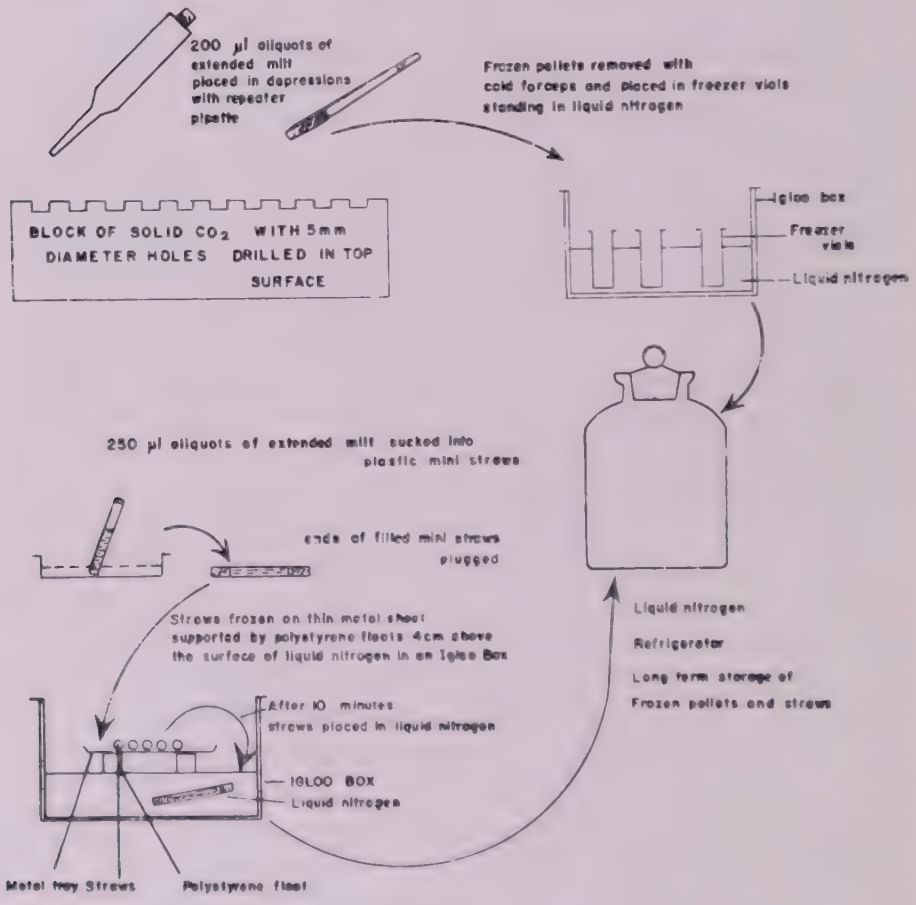
200 µl aliquots of extended milt placed in depressions with repeater pipette
Frozen pellets removed with cold forceps and placed in freezer vials standing in liquid nitrogen
BLOCK OF SOLID CO2 WITH 5mm DIAMETER HOLES DRILLED IN TOP SURFACE
Igloo box
Freezer vials
Liquid nitrogen
250 µl aliquots of extended milt sucked into plastic mini straws
ends of filled mini straws plugged
Straws frozen on thin metal sheet supported by polystyrene floats 4cm above the surface of liquid nitrogen in an Igloo Box
After 10 minutes straws placed in liquid nitrogen
IGLOO BOX
Liquid nitrogen
Metal tray Straws
Polystyrene float
Liquid nitrogen
Refrigerator
Long term storage of Frozen pellets and straws

Fig. 9. Cryopreservation of spermatozoa.

Note: Cryopreservation will be valuable at many stages of genetic improvement. Crossbred and selectively improved stocks may also be subjected to sex and induced triploidy to improve productivity.

c) An alternative method, using only liquid nitrogen, is to fill mini straws of fine plastic tubing with 250 μl aliquots of extended milt, plug the ends and then freeze above liquid nitrogen.

An appropriate cooling rate is achieved by placing the straws of milt on a thin metal tray supported 4 cm above the surface of the liquid nitrogen by a polystyrene float. For this purpose liquid nitrogen can be placed in a polystyrene Igloo box. After 10 minutes the straws should be immersed in the nitrogen and then stored until required. The frozen straws should only be handled with cooled forceps.

d) Once in liquid nitrogen the frozen milt can be stored indefinitely since the rate of deterioration is negligible.

e) Rapid thawing of the frozen milt produces the best fertilization rates.

5 pellets of milt are added to 5 ml of sea water at ambient temperature, shaken for a few seconds until they are slushy and then added immediately to the eggs to be fertilized. Alternatively 2 straws are thawed in a water bath at 40°C for 5 seconds, the ends cut off and the contents added to eggs simultaneously with 5 ml of sea water.

These quantities of milt are probably sufficient to fertilize 500 to 1000 eggs.

Extender solution (Based on Mounib, 1978)

Distilled water	100 ml
Sucrose	4.28 g
$KHCO_3$	1.00 g
Reduced glutathione	0.20 g

Before use add 7 parts of this solution to 1 part of dimethyl sulphoxide. Glutathione is not essential but often improves results.

Modifications

It will be necessary to modify this method to obtain optimal results for each species under investigation. Initially it is essential to

analyse the biochemical composition of the milt, the sperm density etc. and to establish the number of spermatozoa needed per egg, the milt dilution, etc. to give best fertilizations. The toxicity of DMSO to spermatozoa should be established by exposure to different concentrations for 10 minute period without freezing, and then testing the milt in fertilization trials. Factors which may require modification include extender composition, extent of semen dilution, freezing and thawing rates, number of spermatozoa required per egg and dilution of milt during fertilization (see Billard, 1978).

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